THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XVI APRIL, 1935 No. 2

THE TOXICITY OF FILTRATES OF BROTH CULTURES OF MENINGOCOCCI.

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Received for publication January 11th, 1935.

ERLICH, Popowski and Przesmycki (1925) showed that diluted filtrates from 15-day-old broth cultures of meningococci produced erythematous skin reactions when inoculated into the human skin, and that the reacting substance in the filtrates could not be neutralized by mixture *in vitro* with antimeningococcal sera, although injection of antiserum into positive skin reactions sometimes rendered them negative.

Herrold (1927) and Ferry (1931) also noted skin reactions following the inoculation of filtrates of broth cultures of meningococci, and the latter concluded from his experiments on the neutralization of the reacting substance with antitoxic sera that the filtrates contained a diffusable toxin distinct from the " endotoxin " of Gordon (1920) and others.

Krestownikowa (1933) obtained toxic filtrates from broth cultures of Russian strains of meningococci and claimed that these filtrates, besides producing skin reactions on intradermal inoculation, were lethal to mice, guinea-pigs and rabbits on intraperitoneal, intravenous and suboccipital injection. Protection from this toxicity was obtained by the use of both polyvalent and monovalent antimeningococcal sera.

Experiments on these lines were started in Oxford in 1932 and it has been found that toxins lethal to guinea-pigs on subdural inoculation are present in filtrates of 3- to 6-day-old cultures of freshly isolated meningococcal strains grown in standard blood broth.

METHOD OF CULTURE.

Preparation of blood broth.—The broth best suited for the production of toxic meningococcal filtrates is prepared by the method suggested by Wright (1933), the essential point being to effect the reduction of the peptone by

adding it to the chopped meat before boiling. The pH is adjusted to between 6·6 and 6·8 and fresh rabbit heart blood, laked in ether, is added to each flask of the finished medium after it has been autoclaved. The medium is put up in 500 c.c. lots in round flasks, and about 10 c.c. laked blood are added to each flask, after which the medium is incubated at 37° C. for 48 hours to remove the ether.

Inoculation of the medium and preparation of the filtrate.—Each flask of broth is inoculated with the growth from a 24-hour culture of meningococci on a large Dorset egg slope. After 24 to 48 hours' incubation at 37° C. a thics pellicle of growth forms on the surface of the medium, and, on shaking, thik pellicle breaks up and falls in heavy flakes to the bottom of the flask. A new pellicle then forms and is also shaken to the bottom of the flask, thus ensuring rapid growth of the organism. After 4 to 6 days' growth the broth culture is filtered through a Seitz filter and the filtrate is tested for sterility. This filtrate contains the toxin and may be kept in the cold at 4° C. without preservative until required.

THE TOXIC PROPERTIES OF FILTRATES OF ENGLISH STRAINS OF MENINGOCOCCI.

The Skin Reaction.

Intradermal inoculation of filtrates in doses of 0·1 to 0·2 c.c. of the filtrate diluted 1 in 100 to 1 in 1000 produces characteristic reactions in sensitive human subjects. Within 2 or 3 hours an area of erythema spreads from the point of injection ; it reaches a maximum after a further 10 to 18 hours, when it appears as a slightly raised indurated reddened area usually about 30 to 40 mm. across. It then fades rapidly and disappears in about 48 hours from the time of inoculation.

Sensitive subjects react to the inoculation of filtrates from both Group I and Group II meningococci, while others react to neither. Control inoculations of diluted broth do not produce any reaction.

Intraperitoneal Inoculation.

The intraperitoneal inoculation of guinea-pigs and mice with meningococcal filtrates has produced the most varied results. Sometimes a filtrate from a good growth of meningococci with a thick pellicle formation will have no effect on intraperitoneal injection into guinea-pigs of 250 to 300 g. weight, and at other times filtrates from apparently similar cultures of the same strain may prove very toxic. When death occurs as the result of intraperitoneal inoculation of filtrates of English strains of meningococci, the fatal dose is always much higher than that noted by the Russian workers (5 c.c., Krestownikowa, 1933) and titration of the toxicity of a filtrate by this method of injection has not been found possible.

Table I illustrates the great variability of the responses of both guinea-pigs and mice to the intraperitoneal inoculation of filtrates of the same strains of meningococci.

TABLE I.—*Results of Intraperitoneal Inoculation of Meningococcal Broth Filtrates into Guinea-pigs and Mice.*

Strain used for filtrate.	Animals inoculated with filtrates. Number.	Weight (g.).	Dose (c.c.).	Died within 24 hours.	Controls.
GUINEA-PIGS.					
Margeson, Group II	6	200	10	5	Lived.
,, ,,	12	200	10	0	,,
Robinson, Group I–III	6	200	10	3	,,
MICE.					
Margeson, Group II	6	15	2·0	1	,,
,, ,,	5	15	2·0	2	,,

Note.—The dose inoculated was calculated to correspond to the blood volume of the given animal. The control animals were inoculated with corresponding doses of filtered broth.

The figures shown in Table I are examples of a larger series of intraperitoneal inoculations, including the inoculation of filtrates from cultures of strains not mentioned in the table. The results of the inoculation of the Margeson Group II filtrates demonstrate clearly the extremes of variation in toxicity which may occur.

Subdural Inoculation.

The most reliable method of demonstrating the toxicity of the filtrates of meningococcal broth cultures has been found to be the subdural inoculation of guinea-pigs.

Technique of inoculation.—Guinea-pigs of 200 to 1000 g. weight may be used. The animal is deeply anæsthetized with ether, and a mid-line incision about 2 cm. long is made through the scalp between the ears down to the bone. The skin is deflected and the right or left occipital bone is exposed. A fine hole is then bored in this bone lateral to the mid-line and superior to the occipital ridge, until the inner table of the skull is reached. A small hypodermic needle attached to a "tuberculin" syringe containing the inoculum is pressed gently down and forward into the hole until the inner table is pierced. The filtrate is injected slowly and after the withdrawal of the needle the site of inoculation is pressed firmly to prevent the escape of fluid. It is possible in this way to inject the filtrate beneath the dura into the subarachnoid space without damaging the brain or producing any hæmorrhage.

After the injection the skin edges are sewn with silk and the animal is placed in a warm room for the ensuing 48 hours. The animals quickly recover from the effects of the operation and control cases injected with saline or broth appear none the worse weeks later.

The results of subdural inoculation.—The effects of subdural inoculation of meningococcal filtrates into guinea-pigs are rapid and severe. The first sign of a reaction is the quietness and immobility of the guinea-pig, which resents

interference. Within 4 hours of the inoculation the animal exhibits sudden shuddering spasms which shake the whole body every few seconds. These spasms may lengthen into short periods of opisthotonus in which the hind limbs are forcibly extended, or may persist as a coarse tremor until death supervenes. With the commencement of the spasms the breathing becomes very rapid and noisy and a fine froth appears at the external nares, sometimes slightly blood-stained. Death occurs in about 18–24 hours and is preceded by an increasing coldness of the body and a progressive slowing of the heart, the beating of which becomes easily countable. When larger doses of the filtrate have been administered, death may take place in 5 or 6 hours.

As a general rule large guinea-pigs are more susceptible; subdural inoculation of as little as 0·01 c.c. of filtrate will sometimes kill a guinea-pig of 800 g. within 24 hours.

The results of subdural inoculation of guinea-pigs in a large series of experiments on animals of various weights and with filtrates of several strains of both groups of meningococci are set out in Table II.

TABLE II.—*Results of Subdural Inoculation of Filtrates from Broth Cultures of Various Strains of Meningococci into Guinea-pigs.*

Strain used for filtrate.	Animals inoculated with filtrates.			
	Dose (c.c.)	Number.	Weights (g.).	Died within 24 hours.
Scott, Group II (nasopharyngeal)	0·2	10	200–250	8
Farr, Group I–III	0·2	10	200–250	9
Pringle, Group I–III	0·2	9	200–250	8
Robinson, Group I–III	0·1–0·5	57	200–1000	52
Control animals, inoculated with filtered broth	0·1–0·5	34	200–1000	0

Note.—The above results do not include experiments with dried filtrates, "antitoxic" sera, etc.

The subdural inoculation of filtrates of sterile blood broth, or of filtrates of broth cultures of organisms such as *B. coli*, has no effect on guinea-pigs. On the other hand it has been found that filtrates of broth cultures of some of the other Gram-negative cocci, *e.g. N. gonorrhœa*, *N. pharyngis* and *M. catarrhalis*, may be lethal to guinea-pigs when injected subdurally in doses of 0·4 c.c. This is being further investigated.

It is possible to kill 3000 g. rabbits with large subdural inoculations of the filtrates, but it is better for this purpose to use the concentrated toxin, precipitated from the filtrates by acetone. Inoculations of 0·001 g. of this substance dissolved in saline will kill a 3000 g. rabbit in about 18 hours. The rabbit exhibits much the same signs as the guinea-pig, the respiration being affected early. Spasms are less frequent and more prolonged. The white cell blood-count falls rapidly in the first few hours, being frequently less than 50 p.c. normal after 5 hours.

PATHOLOGICAL CHANGES PRODUCED BY THE FILTRATES.

General Post-mortem Changes.

The filtrates act in general as a tissue poison affecting mainly the blood-vessels, which are greatly distended and damaged in many of the organs.

Abdominal viscera.—The intestines are congested; the colon is usually empty of fæces and its lumen contains mucus mixed with desquamated epithelial cells and red blood-cells. The kidneys are congested and bleed on cutting. The cortical vessels are greatly distended with blood, especially in the region of the glomeruli.

The suprarenals are deep red in colour and darker spots of hæmorrhage may be seen under the capsule. The vessels of the capsule and throughout the cortex are distended and small areas of hæmorrhage appear amongst the cortical cell columns and in the zona reticularis. The medullary vessels are usually full of blood, but occasionally the central vein may be empty. The suprarenal changes occur in all degrees of severity and are probably due to the action of the toxin on the vessels, rather than to any specific action on the gland.

The liver is deeply congested. Small scattered punctate hæmorrhages are frequently found over the greater curvature of the stomach.

The appearance of the spleen is characteristic. It is invariably very pale and soft and the corpuscles show up plainly on the surface. There is no enlargement. The vessels of the capsule are empty, as are the splenic arteries, and there is very little free blood in the pulp. The veins are full of blood containing relatively fewer leucocytes than normal. The splenic nodules are prominent and there is an increase of lymphocytes in the substance of the organ.

Thoracic viscera.—The heart is usually in a state of ventricular contraction whilst the auricles are dilated. The lungs show mottling of the surface with small hæmorrhages and there is a great degree of stasis and congestion at the bases. The bronchi are full of blood-stained frothy mucus which often extends up the trachea. The bronchial mucosa is desquamated in places and the lumen is filled with debris and red blood-cells. The vessels are greatly dilated and many of the alveoli are packed tightly with red blood-cells, so that the appearance in some cases resembles early red hepatization.

Pathological Changes in the Brain.

No injuries to the brain or hæmorrhages under the dura have followed the subdural inoculation of the toxic filtrates. Where death occurs after the inoculation the surface vessels of the brain are congested and dilated. The whole brain has a pinkish colour and on cutting into it, hæmorrhages may sometimes be seen in the substance, usually beneath the floor of the lateral ventricles or in the pons. There is no obvious pus, but the meninges frequently have a slimy feel, as though they were covered with a thin layer of cells.

Microscopically, the superficial vessels of the cortex are found to be dilated and full of blood containing large numbers of leucocytes. The cisternæ and sulci are packed with a loose mixture of lymphocytes and polymorphonuclear

leucocytes, which spread as a thin layer over the brain surface. Within the substance of the brain the nervous tissue appears normal, but profound changes are visible in the deep blood-vessels, especially those running along the floor of the lateral ventricles and those in the pons. Here the vessels are thickly lined with layers of mixed leucocytes which sometimes appear to have infiltrated the vessel wall and surrounding tissue, producing a cuffing of the vessel. In places the vessels appear to have burst and small hæmorrhages have developed round them in the brain substance. This is most common in the region of the pons and mid-brain. Extensive hæmorrhages may sometimes be seen also beneath the floor of the lateral ventricles.

The changes in the brain and meninges may be seen in Figs. 1, 2 and 3. They occur constantly in the brains of animals killed by subdural inoculation of the meningococcal filtrates, but they are not seen in animals which have succumbed to intraperitoneal injection. The post-mortem changes in the abdominal and thoracic viscera are, however, found in animals after both subdural and intraperitoneal inoculation. It is possible, therefore, that the toxin may be able to pass from the central nervous system to the blood-stream but not in the reverse direction.

THE NATURE OF THE MENINGOCOCCAL BROTH TOXIN.

The physical examination of the toxin present in the filtrates of broth cultures of meningococci shows it to be a very stable substance. This stability has also been noted by Ferry (1931) and Krestownikowa (1933).

It retains its toxic properties after boiling for an hour. Animals react in the same way to inoculations of either boiled or fresh toxin filtrates. The post-mortem findings are the same in both cases. Storing at room temperature or at 4° C. for weeks does not affect the toxicity of the filtrates.

It is possible to precipitate from the filtrate by means of acetone (after the method of Wadsworth and Quigley (1931) for concentration of streptococcal toxin) a yellow flakey substance, which when taken up in half the original volume of saline and injected into guinea-pigs subdurally is as toxic as the original filtrate.

The toxin is produced rapidly in broth cultures, being in evidence after the organisms have been growing for only 48 hours. Filtrates from 3-day-old cultures are as toxic as those obtained from 6-day-old cultures.

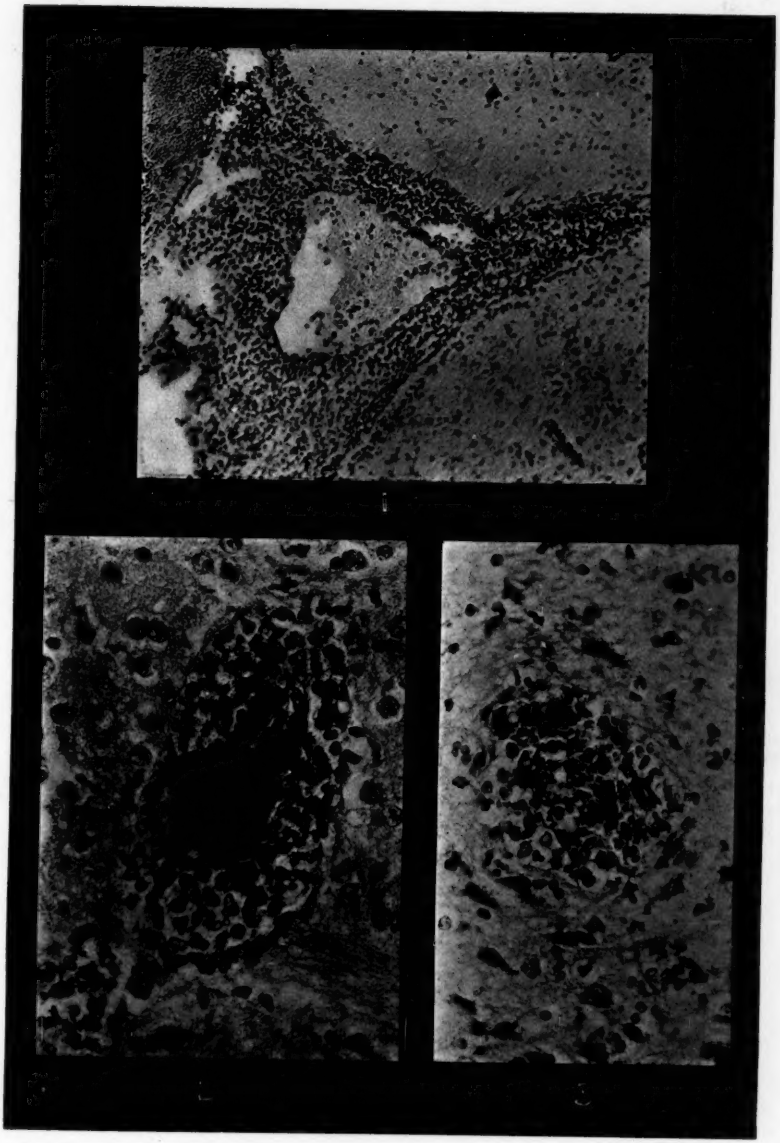
There is a close relation between the rate of production of the toxic

DESCRIPTION OF PLATE.

FIG. 1.—Section through a sulcus of brain of guinea-pig killed by subdural inoculation of filtrate of meningococcal broth culture. Note the dilatation and distension of vessels and the accompanying collection of large numbers of polymorphonuclear leucocytes and lymphocytes in the meninges.

FIG. 2.—Section through the pons of a guinea-pig killed by subdural inoculation of meningococcal broth filtrate. Note the lining and cuffing of the vessel with polymorphonuclear leucocytes and lymphocytes.

FIG. 3.—Section through the pons of a guinea-pig killed by subdural inoculation of meningococcal broth filtrate, showing small hæmorrhage into the brain substance from a ruptured vessel.



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substances in the broth cultures and the appearance of the specific soluble carbohydrate substances derived from the destruction and autolysis of the meningococcal cells. Thus, filtrates from 3-day-old cultures, which are already highly toxic, will produce precipitation at a dilution of 1 in 1,000,000 with monovalent antimeningococcal precipitin sera, whereas filtrates from 24-hour-old cultures will not precipitate with the specific sera at dilutions greater than 1 in 100.

The intraperitoneal and subdural inoculation of guinea-pigs with toxic substances or " endotoxins " derived from the disintegration of meningococcal cells, such as those prepared by Riley and Wilson (1932) and Malcolm and White (1932), or with living meningococci (Flexner, 1907), produces reactions which are very similar to those produced by inoculations of the toxic filtrates. There are, however, certain differences. In the brain, for example, the leucocytes found lining and cuffing the vessels and infiltrating the meninges following an inoculation of " endotoxin " are all polymorphonuclear leucocytes, in contrast to the mixture of lymphocytes and polymorphonuclear leucocytes found after an injection of the toxic filtrates. Again, the spleens of animals killed by endotoxic extracts are not pale and empty of blood as they appear after the inoculation of the toxic filtrates, but are full of blood.

SUMMARY.

Filtrates of 3- to 6-day-old cultures of meningococci of both Groups I–III and II contain substances toxic to guinea-pigs, mice and rabbits.

These toxic substances are best demonstrated by subdural inoculation of the animal through the occipital bone into the subarachnoid space.

The action of the filtrates on animals is very similar to that of various " endotoxins " produced directly from meningococcal cells. There are, however, histological differences which suggest that the toxic filtrates contain substances not present in the cell extracts.

Protection experiments are in progress.

I desire to thank Dr. A. D. Gardner, Standards Laboratory, Oxford, for his interest in the work, and Dr. G. F. Petrie, Lister Institute, Elstree, for the loan of sections of the brains of guinea-pigs killed by intradural inoculation of meningococcal " endotoxin ".

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THE MODE OF FORMATION OF AGGREGATES IN BACTERIAL AGGLUTINATION.

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Received for publication January 22nd, 1935.

THE current conception of the mechanism that determines the mutual flocculation of antigen and antibody in the presence of electrolytes is, in general terms, as follows :

The antibody unites specifically with the antigen, the union depending on the presence and arrangement of certain active chemical groupings on the two reagents. This specific union is often spoken of as the first stage of precipitation or agglutination. As a consequence of this union, the antibody globulin that has become attached to the antigen acquires new properties ; it may, as a result of the occupation or occlusion of certain hydrophile groupings, become preponderatingly hydrophobe, or it may be altered in other ways. In any event the result is to render the antigen-antibody compound sensitive to the flocculating action of electrolytes, of a kind and in a concentration to which uncombined antigen or antibody is indifferent. It is commonly held that the most important effect of the electrolyte is to lower the surface charge of the primary antigen-antibody particles, and so to allow their aggregation into particles or floccules of increasing size. This is often spoken of as the second stage of precipitation or agglutination. It is commonly regarded, in contrast to the first stage, as being non-specific, the primary antigen-antibody particles behaving as a salt-sensitive colloidal suspension.

In his recent monograph on the chemistry of antigens and antibodies Marrack (1934) has advanced an alternative hypothesis. He suggests that the antigen and antibody molecules may form a complex lattice, the form of the lattice depending in part on the ratio of antigen to antibody in the reacting mixture. On this view the visible particles or flocculi in a precipitating mixture would not be non-specifically-formed aggregates of a primary antigen-antibody compound, but masses of antigen and antibody molecules bound together by specific linkages, any one antigen molecule within the mass being united to two or more molecules of antibody, any one molecule of antibody to two or more molecules of antigen. The antibody would thus play the part of Ehrlich's amboceptor, or more correctly of a polyceptor, except that it would be binding one antigen molecule to another, not red-cells or bacteria to complement. This conception differs sharply from that outlined above, in that the second stage, the formation of the larger aggregates, is as specific as the first, the initial union of antigen to antibody. It would, perhaps, be more correct to say that it abolishes any real distinction between the two stages postulated by the older view.

Marrack's hypothesis has certain very definite advantages over the two-stage conception in that it affords a simpler explanation of the inhibitory

effect of antibody excess in precipitation and agglutination reactions, and of the properties of simple and complex haptens. Its acceptance would, as Marrack has emphasized, involve the assumption that electrolytes play some *rôle* in determining the size and nature of the aggregates formed; but this leads to no inherent difficulty. It is not, however, our purpose to discuss the possible significance of this hypothesis, but to record a few observations that seem to us to yield it experimental support.

If, in a reacting mixture, we have two antigens and two corresponding antibodies, the two antigens possessing no common active grouping, the nature of the particles or floccules formed should differ on the two hypotheses sketched above. With antigens G_1 and G_2 and antibodies A_1 and A_2, accepting the view that the reagents may unite in varying proportions, the primary particles will, on either view, be $G_{1m}\,A_{1n}$ and $G_{2x}\,A_{2y}$. In the second stage, on the older view, these primary particles will unite more or less at random, under the influence of electrolyte, giving larger aggregates $(G_{1m}\,A_{1n})_p\,(G_{2x}\,A_{2y})_q$. On Marrack's hypothesis this secondary non-specific aggregation will not occur, or will at least be postponed until a later stage, and large aggregates will be formed of the type $G_{1m}\,A_{1n}$ and $G_{2x}\,A_{2y}$, the form of the lattice in each case, and thus the value of m, n, x and y depending on the ratio of G_1 to A_1 and G_2 to A_2 in the reacting mixture. If then we found that, in a complex reacting mixture of this kind, relatively large aggregates were formed, each consisting entirely of one kind of antigen and its corresponding antibody, our results would accord better with Marrack's hypothesis than with the older view.

In the precipitin reaction there is, at the moment, no obvious way of determining the distribution of two or more antigens among the different particles formed; but the considerations outlined above should apply in the case of bacterial agglutination, and here it is possible, in various ways, to determine with reasonable accuracy the composition of the clumps or floccules. This is what we have done.

EXPERIMENTAL.

Our experiments have been extremely simple. We have taken various combinations of two antigenically different bacteria and the corresponding antisera, allowed them to react in a single mixture, and observed under the microscope the nature of the aggregates formed. It has not proved altogether easy to obtain satisfactory results; and though no useful purpose would be served by describing details of technique, a few points may be noted.

It is necessary, as a preliminary to such experiments, to carry out cross-agglutination tests, with a range of antiserum dilutions covering a concentration considerably higher than that used in the experiment itself, in order to ensure the complete absence of common antigenic components. It is necessary, also, to adjust the mixture with some care in accord with the time-relations of the two reacting systems. If, for instance, the agglutination of bacterium A by antibody *a* were much more rapid than the agglutination of bacterium B by antibody *b*, we might find separate A and B aggregates, even though the larger aggregates were formed non-specifically. We have attempted to meet this criticism by setting up optimal proportions tests, with varying dilutions of antiserum, and selecting dilutions for our actual experiments

such that agglutination of the two kinds of bacteria in the mixture will occur synchronously. It has not, however, been possible to secure ideal conditions in this respect. The fact that two reacting systems reach the stage of visible agglutination at approximately the same moment does not necessarily imply that flocculation, from that point onwards, will proceed at the same rate. All we feel justified in claiming is that in our reacting mixtures each of the two types of bacteria present have been undergoing progressive aggregation into larger and larger clumps over the same period of time, and that this period has lasted sufficiently long (many minutes to a few hours) for the small clumps of one bacterium to adhere to the small clumps of the other, had there been any tendency for them to do so.

As regards the method of microscopical observation, it is possible, using morphologically distinguishable bacteria, to observe the whole process on the slide under dark-ground illumination. We employed this method in some of our earlier experiments, but found it unsatisfactory (*a*) because it is not easy, with a layer of fluid sufficiently thick to allow of free movement of the bacteria, to be certain of the composition of the larger clumps, and (*b*) because it is less easy than by the second method to examine many different specimens within a short space of time.

The method that we have employed in the great majority of our experiments has been as follows : We have taken two bacteria A and B, showing no antigenic relationship, and with sufficient morphological difference to enable them to be distinguished from each other when in fairly large aggregates. From cultures of these we have prepared saline suspensions, usually killing the bacteria by the addition of 0·25 p.c. formol. These suspensions have been standardized by opacity to contain the same number of organisms per c.c. A mixture of equal parts of these suspensions has been placed in a small tube, and to it has been added an equal volume of a mixture of the two corresponding antisera, *a* and *b*, so diluted that agglutination of A and B will occur over approximately the same time-interval. The tubes have sometimes been left at room temperature, sometimes incubated at 55° C. in a water-bath, according to the system with which we have been working. At varying intervals thereafter we have withdrawn a sample of the reacting mixture with a capillary pipette, mixed a drop on a slide with a drop of some convenient stain, usually a 1/10 dilution of methylene-blue or borax-blue, covered the drop with a glass slip, and examined at once under a 1/12th objective. Alternatively we have in some cases allowed agglutination to occur in tubes of 2·5 mm. bore standing in the test-tubes containing the mixture, and expelled a drop on to a slide at suitable intervals. This involves less breaking of loose clumps than does the use of a capillary pipette. With bacteria that show sufficient morphological differences this method has proved entirely satisfactory. We have, in every case, set up control tubes containing each antiserum in the absence of the other, and in these the sensitized organism has always agglutinated, while the other has remained dispersed.

It would be of obvious advantage to apply a differential stain, so that one could work with organisms having a similar morphology. The use of Gram-positive and Gram-negative organisms followed by the differential staining of dried films of the agglutinated bacteria gave poor results, nor was the range

of organisms available greatly increased by this method. A technique that appeared more hopeful was to stain the organisms in bulk, using different coloured stains, prepare mixed suspensions of the differently stained organisms, and then submit them to agglutination. This method has, however, in our hands presented formidable technical difficulties. To stain the organisms sufficiently deeply for this purpose it is necessary to employ high concentrations of stain, and every stain we have employed has tended to agglutinate the bacteria in concentrations not much greater than those it has been necessary to employ. The stain diffuses from the bacteria rather rapidly, and in the reacting mixture each bacterium takes up some of the stain that has diffused from others, so that the differentiation tends to be lost. We have the impression that this diffusion is more rapid in bacteria that have been sensitized with antibody than in unsensitized organisms. We have obtained a few fairly satisfactory results with this technique, with controls that appear to eliminate the more obvious sources of error ; but we have not yet developed a reliable method along these lines, and we should regard the results obtained by the second method outlined above as supplying almost all the significant evidence that we have as yet obtained.

In the experiments that we desire to record in this paper the following combinations of organisms were used.

(1) Pneumococcus Type XXXI with *Bact. typhosum* (O).
(2) ,, ,, ,, ,, *stanley* (H).
(3) ,, ,, ,, ,, *enteritidis* (O) + (H).
(4) ,, ,, ,, ,, *flexneri*.
(5) ,, ,, ,, Pneumococcus Type I.

The first four combinations were studied by the second method, the fifth by preliminary staining with methylene-blue and cresoidin.

The results are sufficiently illustrated by Figs. 1–6.

Fig. 1 shows the aggregates formed in a mixture containing Pneumococcus Type XXXI and *Bact. typhosum*, with a Type XXXI antiserum and a serum prepared against *Bact. enteritidis*, which shares the *Bact. typhosum* somatic antigen. In the upper half of the field is an aggregate of pneumococci, in the lower half a tightly-packed aggregate of typhoid bacilli. There is no evidence of mixture.

Fig. 2 shows the aggregates formed in a mixture containing Pneumococcus Type XXXI and *Bact. stanley*, in which the anti-enteritidis serum was replaced by anti-typhoid serum containing the antibody corresponding to the flagellar Stanley antigen. In the upper half of the field is an aggregate of pneumococci, in the lower half a loosely-packed clump of Stanley bacilli of the characteristic flagellar-agglutination type.

Fig. 3 shows the aggregates formed in a mixture of Pneumococcus Type XXXI and *Bact. flexneri* with the corresponding antisera. This particular strain of *Bact. flexneri* is highly pleomorphic and easily recognizable from its appearance. To the left and above is an aggregate of pneumococci ; to the right and below is an aggregate of Flexner bacilli.

Fig. 4 shows the aggregates formed in a mixture of Type I and Type XXXI pneumococci with the corresponding antisera. Before setting up the agglutinating mixture the Type XXXI pneumococci were stained with methylene-blue, the Type I pneumococci with cresoidin. It will be noted that the

cresoidin-stained cocci have a granular appearance. The differentiation is assisted by the fact that the capsules of the Type XXXI pneumococci are here much better developed than those of the Type I organism. Above is an aggregate of Type XXXI cocci, below a small aggregate of Type I cocci.

The objection might reasonably be raised that the use of the different stains might so affect the cocci as to interfere with their adherence. A suspension of Type XXXI pneumococcus was therefore divided into two parts, one of which was stained with methylene-blue, the other with cresoidin. These suspensions were mixed, and to the mixed suspension a Type XXXI antiserum was added. Aggregates were rapidly formed consisting of cresoidin-stained and methylene-blue-stained cocci distributed at random (Fig. 5).

So far as our experience has gone it has been entirely consistent. When mixtures of the kinds described above are allowed to react, pneumococci adhere to pneumococci, but not to typhoid bacilli, typhoid bacilli to typhoid bacilli, but not to pneumococci, and so on. Whether the large aggregates so formed tend, at a later stage, to adhere to one another as the result of non-specific factors we are unable to say with any certainty. Fig. 6 shows a large tight clump of pneumococci in immediate proximity to a large loose clump of typhoid bacilli, and at the interface between the two aggregates there is certainly some intermingling of the two organisms. Appearances of this kind are not uncommon in specimens withdrawn from a reacting mixture during the later stages of agglutination. Whether they result from a true non-specific adherence or whether they are due to the mechanical juxtaposition of these large aggregates in the preparation of the slide is, we think, impossible to determine.

DISCUSSION.

It seems to us that these findings, so far as they go, accord well with the lattice hypothesis put forward by Marrack, but are difficult to explain on the current view that flocculation occurs in two stages, the first specific, the second non-specific. Quite clearly they do not yet go far enough, and their real significance can only be assessed in the light of much more extensive experience.

Equally clearly, of course, the demonstration that the formation of antigen-antibody floccules is throughout a specific process would not alter the fact that a single bacterium or red-cell, sensitized by its homologous antibody, exhibits towards various agents a non-specific behaviour that is different from that of the non-sensitized cell, as, for instance, in its lysis by serum complement or in its phagocytosis by leucocytes or macrophages.

It would, however, seem desirable that the interesting implications of Marrack's hypothesis should be given due weight in future attempts to determine the mechanism of those antigen-antibody reactions in which flocculation plays a part.

We should wish to express our thanks to Mr. W. Bale for his assistance in taking the microphotographs.

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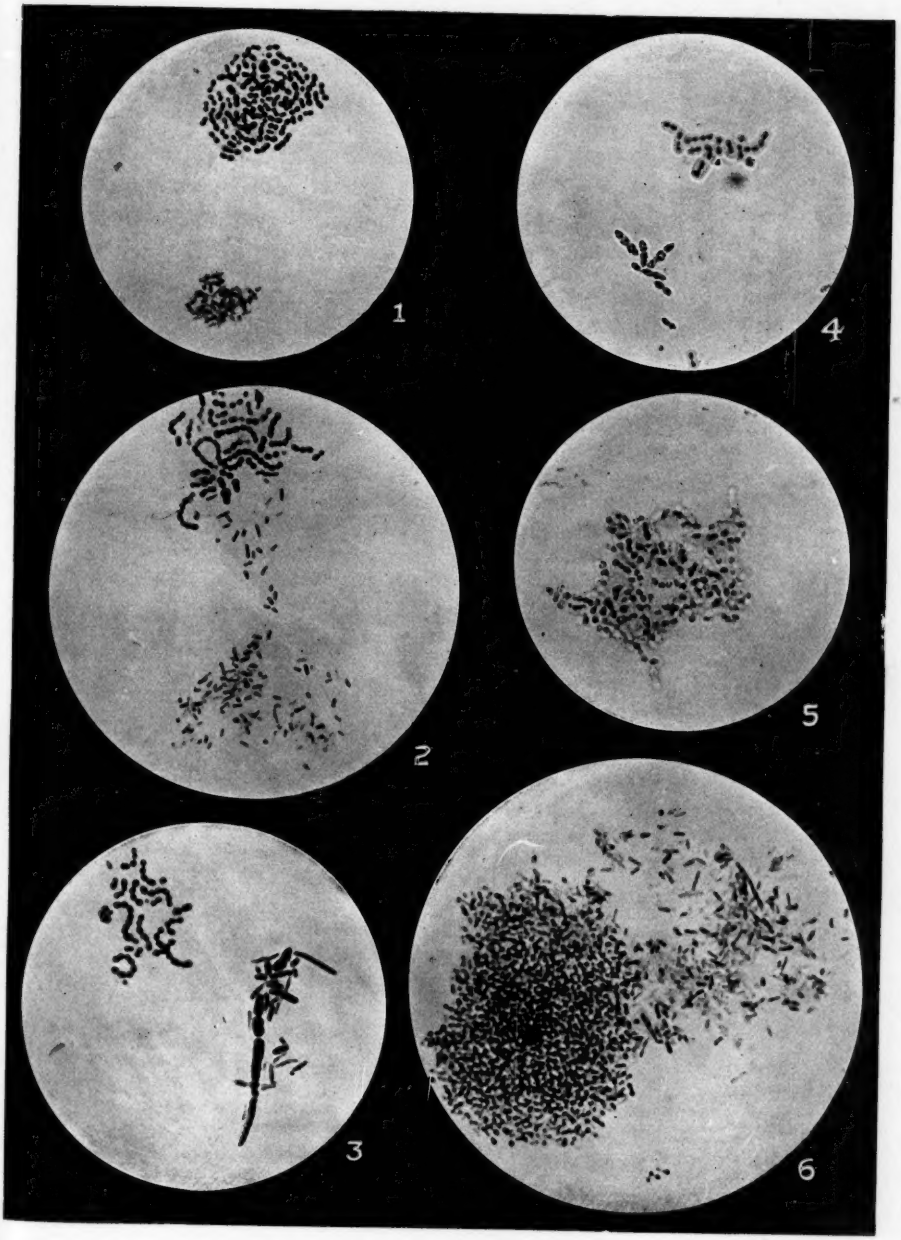

Topley, Wilson and Duncan.

THE EFFECT OF INTERFERENCE WITH THE VASCULAR SUPPLY ON THE INDUCTION OF DIBENZANTHRACENE TUMOURS.

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Received for publication January 22nd, 1935.

It has been previously reported (Orr, 1933) that the induction of tumours of the skin in mice by coal tar was appreciably accelerated by two procedures interfering with the normal vascular supply. In one experiment subcutaneous fibrosis was produced by the preliminary insertion of sutures into the tissue underlying the area to be tarred; in the other adrenaline hydrochloride was injected at weekly intervals into the same region. Tumours were present in half the survivors in 11 and 13 weeks respectively, while this level was not attained in the control groups until after 17 weeks. A less convincing result was obtained with ephedrine sulphate, the corresponding length of time being 15 weeks, while a group injected with physiological saline took 16 weeks. The incidence of malignancy at the 21st week appeared to be significantly raised by adrenaline, and after the preliminary fibrosis by sutures, but not by ephedrine.

In the present communication the results are given of similar observations on experimental carcinogenesis with 1 : 2 : 5 : 6-dibenzanthracene. Apart from the obvious advantages of carrying out an experiment of this type with a pure chemical substance rather than the heterogeneous mixture which constitutes tar, the time taken by dibenzanthracene for the induction of tumours is considerably longer than that required in the case of tar. It seemed reasonable to hope that this longer latent period would offer more opportunity for a decisive result when an acceleration of the process of tumour induction was under study.

EXPERIMENTAL.

The experimental procedure was similar to that employed in the tar investigations. A group of 50 white mice was used for each experiment. The hair over a small area of skin in the interscapular region was clipped close, and the injection of ephedrine or adrenaline was made immediately prior to applying the dibenzanthracene solution. A saturated solution in benzene of 1 : 2 : 5 : 6-dibenzanthracene was used, and applied with a medium-sized camel-hair brush. Applications were made at weekly intervals. The injections consisted of a 2·5 p.c. solution of ephedrine sulphate, and adrenaline hydrochloride solution (B.P.) diluted tenfold immediately before use; 0·05 c.c. of each was injected (*i.e.* 1·25 mg. ephedrine and 0·005 mg. adrenaline). The needle was

introduced at the caudal extremity of the treated area of skin and passed intracutaneously to its centre, the object being to standardize as far as possible the site of trauma, lest this should have any effect in the neoplastic process.

The control group of mice were clipped and treated with dibenzanthracene weekly in an exactly similar way, except that no injection was given. The fourth group was derived from 60 mice, in which subcutaneous scar-tissue had been produced. The hair was clipped and then, with aseptic precautions, linen thread sutures were passed under the skin four times in different directions, all needle punctures being kept outside the boundary of the area later to be treated with dibenzanthracene. The sutures were left *in situ* for five weeks, and then removed. Any mice in which ulceration had taken place were discarded, and 19 days after the removal of the sutures (*i. e.* when healing was complete) weekly applications of dibenzanthracene were started on the remaining 49 mice. It has previously been indicated that this procedure results in the production of acellular fibrous scar-tissue in the subcutis, and for convenience this group will be referred to as the " fibrosis " group.

The results are shown in Table I. The figures for two groups of controls and two adrenaline groups have been combined for brevity. Regression of tumours not uncommonly occurs, and was most frequent in the adrenaline and ephedrine groups. Tumours which regressed have been excluded from the records. It will be seen that there is quite a definite acceleration of the process of tumour induction in the adrenaline and " fibrosis " groups, in which eight and five tumours respectively had appeared by the time the first control wart was found, although the control group of mice contained many more survivors than the other groups. The time taken to produce tumours in 50 p.c. of the surviving animals in any group is probably the best single criterion, and this occurred 8 and 4 weeks before the controls in the adrenaline and " fibrosis " groups respectively. It seems not improbable that it would have been still earlier in the latter group had it not been for the deaths of some of the tumour-bearing animals. The ephedrine group gave a result substantially the same as the controls.

The rate of appearance of tumours encountered in the control group is similar to that recorded by other workers. The largest series is that of Cook, Hieger, Kennaway and Mayneord (1932), who obtained 107 tumours out of an initial total of 273 mice. Two-thirds of these tumours first appeared in the 7th, 8th and 9th months. As the majority of the tumour-bearing mice were killed as soon as invasion of muscle was suspected, it is not possible to estimate exactly a period corresponding to that of the present 50 p.c. level, but a rough calculation based on detailed graphs respecting 233 of their animals suggests that it would have been somewhere between the 30th and 35th weeks. Seelig (1934) used 127 albino mice, and states that the first tumours were observed about the 18th week, but that it was not until the 22nd to 24th week that papillomata were noticeable, when they began to develop in appreciable number; more exact figures are not given, but these data are in agreement with those of the present control series. A small series is reported by Maisin and Liégeois (1933), who had 11 survivors with 5 tumours after 20 weeks, and 6 survivors with 4 tumours in 23 weeks ; this is an earlier incidence than has been obtained by other workers, but the very small figures reduce its statistical significance.

TABLE I.

Weeks.	Dibenzanthracene only.			Dibenzanthracene + ephedrine sulphate.			Dibenzanthracene + adrenaline hydrochloride.			Dibenzanthracene + "fibrosis".		
	Survivors.	Tumours.	P.c.	Survivors.	Tumours.	P.c.	Survivors.	Tumours	P.c.	Survivors.	Tumours.	P.c.
0	100	..	..	50	..	..	100	..	..	49	..	..
7	78	..	..	34	..	..	38	..	..	45	1	2
10	65	..	..	33	..	..	38	1	3	42	2	5
13	65	1	2	32	..	..	37	8	22	31	4(+1)	13
16	60	2	3	31	1	3	36	12	33	26	6(+1)	23
19	56	6	11	30	3	10	35	18	51	22	4(+3)	18
23	45	13	29	26	6(+1)	23	34	23	68	18	10(+4)	56
27	39	20(+3)	51	23	9(+3)	39	33	26(+1)	79	13	7(+7)	54
28	39	20(+3)	51	22	11(+3)	50	32	26(+1)	81	12	8(+8)	67

The figures in parentheses indicate the number of tumour-bearing animals which had died before the date indicated.

The earliest tumour recorded by Kennaway *et al.* appeared on the 64th day, and only 4 of their 107 tumours had occurred up to the 18th week ; the first tumour of Seelig was found at 18 weeks, and that of Maisin and Liégeois at 84 days. It therefore seems justifiable to believe that the present adrenaline and " fibrosis " groups with 12 tumours out of 36 survivors and 6 out of 26 respectively after 16 weeks, represent a more rapid induction of tumours than has been obtained by the simple application of dibenzanthracene. It also appears that the first two tumours which appeared in the " fibrosis " group, at 7 and 8 weeks severally, are the earliest dibenzanthracene tumours so far recorded.

The experiments were terminated at the end of 31 weeks by killing the mice and examining the tumours histologically to determine the proportion of malignancy at that date. Only those tumours which infiltrated the panniculus carnosus were classified as malignant ; any others which on general histological grounds appeared malignant were termed doubtful. The result is shown in Table II. The total number of malignant tumours is so small that no conclusion is permissible. It would have been better to have selected a later date for the histological examination, but the mice were killed at 31 weeks because an animal in the adrenaline series which died at 27 weeks showed a carcinoma with metastases in lymph-glands and lungs, and gave the impression that the tumour itself was the cause of death.

TABLE II.—*Histology of Tumours : Mice killed at 31st Week of Experiment.*

	Malignant.	Doubtful.	Benign.	Percentage of total survivors showing malignancy.
(*a*) Dibenzanthracene only . .	1	4	18	3
(*b*) ,, + ephedrine .	1	2	8	5
(*c*) ,, + adrenaline	4	2	18	14
(*d*) ,, + " fibrosis "	3	2	4	25

In addition, a mouse of series *c* which died at 27 weeks had a malignant tumour with metastases in lymph-glands and lungs. There were no other malignant tumours in the incidental deaths during the experiment.

The malignant tumours included squamous-celled carcinomata with cell-nests and keratinization, but dedifferentiated types were more frequent than with tar. In one case it was found impossible to decide between spindle-celled carcinoma and leiomyosarcoma, though the former diagnosis was preferred on the grounds that similar areas could be found in other tumours, which also showed typical carcinoma in places. In three of the ten tumours it was impossible to trace evidence in the tumour itself of an origin from squamous epithelium ; in others such evidence was only present in some parts.

As the adrenaline solution used also contained a small amount of hydrochloric acid and chlorbutol, it was deemed advisable to carry out a small experiment to exclude the possibility of attributing the effect to these substances. A solution of precisely the same composition except that the adrenaline was left out was used and the result is given in Table III. It will be seen

that the adrenaline group behaved as in the previous experiment, while the rate of incidence of tumours in the HCl-chlorbutol group was substantially the same as that in the original controls.

TABLE III.

Weeks.	Dibenzanthracene + adrenaline hydrochloride.			Dibenzanthracene + same solution without adrenaline.		
	Survivors.	Tumours.	Percentage.	Survivors.	Tumours.	Percentage.
0	50	..	..	50	..	..
11	12	1	8	18	..	..
16	12	5	42	18	1	6
20	11	6	55	18	3	17
28	10	8(+1)	80	14	7	50

DISCUSSION.

While the results here presented are based on rather small groups of animals, they derive importance in that they tend to support the previous results with tar (Orr, 1933). In view of the uncompromisingly negative result with ephedrine, it would seem that the doubtful result obtained with injections of ephedrine during the application of tar should be disregarded. In the case of adrenaline and "fibrosis", the results with dibenzanthracene agree with the tar results in that a sensible degree of acceleration of tumour induction is obtained by the use of these agents.

It was suggested in the earlier paper that, as the application of tar results in fibrous thickening of the corium and subcutis, which may lead to interference with the vascular supply of the overlying skin, one factor in the origin of tumours might be the ischæmic conditions thus brought about. Similar changes are found when dibenzanthracene is the carcinogenic agent employed, but they develop more slowly. This provokes the suggestion that herein lies the explanation of the longer latent period before the appearance of the dibenzanthracene tumours. An objection to the view that the carcinogenic action of either tar or dibenzanthracene is due solely to a direct effect on the epithelial cells lies in the prolonged course of applications which appears to be necessary to produce tumours. It therefore seems inadvisable at the present time to dismiss the possibility that the carcinogenic action of these substances may, at any rate in part, be related to the changes produced in the deeper tissues.

SUMMARY.

1. Fibrosis of the subcutaneous tissue, produced by the insertion and subsequent removal of linen threads, resulted in an acceleration of tumour induction in mice by means of 1 : 2 : 5 : 6-dibenzanthracene.

2. Weekly injections of adrenaline hydrochloride (B.P.) under the treated epithelium led to a more rapid appearance of dibenzanthracene tumours. The effect appears to be due to the adrenaline, and not to any other constituent of the solution.

3. Ephedrine sulphate had no effect on the rate of induction of tumours by dibenzanthracene.

4. The incidence of malignancy after 31 weeks of experiment is given.

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CONCENTRATED ANTI-PLAGUE SERUM.

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Received for publication February 5th, 1935.

SERUM treatment of plague would still appear to be the best method available. Successful results have been recorded outside India, although in India itself the results have not been so satisfactory (Naidu *et al.*, 1931). The reason for this is probably connected with the admittedly greater virulence of *B. pestis* in India than elsewhere.

Taylor (1933) states that "used in the large doses which appear to be necessary the serum tends to have a cardio-depressant effect in the serious septicæmic type of plague case. The removal of protein fractions other than those with which the causative (? misprint for curative) value is associated is undoubtedly called for".

Serum Preparation at the South African Institute for Medical Research.

In the Institute serum is produced by the intravenous injection of horses, over a long period of time, with emulsions of local strains of *B. pestis* killed by heat. The strains employed are as virulent as it has been possible for us to obtain, but we fear they would not be accounted highly virulent by the Indian standard. We have never obtained a strain locally with virulence approaching that of the Indian strains and even a culture of the strain used for the production of the Haffkine vaccine which the Director of the Haffkine Institute was kind enough to send us, had fallen to the African standard on arrival and no amount of passage through rats improved it.

Method of Concentration.

The method adopted for the concentration of plague serum is similar to that employed in the case of some other antibacterial sera, antitoxic sera and

antivenene. It is a modification of the Brunner-Pinckus fractional salt precipitation process employing sodium sulphate, and full practical details will be found in a publication by one of us on the concentration of antivenom serum (Grasset, 1932). Briefly, the method consists of two additions of anhydrous sodium sulphate to serum which has been heated to 55° C.; the first addition gives a sodium sulphate concentration of 11·5 p.c., resulting in a precipitation of the euglobulin fraction, which is discarded. The addition of a further 6·5 p.c. sodium sulphate to the filtrate precipitates the pseudoglobulin, the fraction which contains the majority of the plague antibodies. The yield is approximately one-tenth of the original unconcentrated serum, the average protein content being about 18 p.c.

Method of Testing the Serum.

The protective qualities of the serum were gauged by testing its power to prevent or cure infections in rats experimentally inoculated with live virulent *B. pestis*. The inoculations ot *B. pestis* were given subcutaneously and the serum was administered intraperitoneally. The rats employed were wild rats trapped in Johannesburg, all of the species *R. rattus*, the varieties *frugivorus* and *alexandrinus* being represented in about equal numbers. The variety *R. rattus rattus* was not represented, it being very seldom seen locally. One M.L.D. of the infecting organisms was taken as that number which could be relied on regularly to kill a rat in 3, or at most 4 days.

The serum was not used in its most highly concentrated form, but diluted with an equal volume of saline solution. In the protocols of the tests, therefore, " concentrated " serum means fully concentrated serum diluted 1 : 2.

The tests were all made in three parallel series—one set of rats getting concentrated serum, one set " ordinary " serum (the serum from which the concentrated had been made), and one set as controls getting no serum. The sera were given either 24 hours before inoculation, at the same time as inoculation, or at some period or periods after inoculation. Over 250 rats were treated in one or other of these ways, exclusive of the untreated controls.

Through consideration of space full protocols of the tests are not given, but the three reported below may be taken as representative of the whole.

In the first trials the ordinary serum and the concentrated serum were used in equal quantities, but it soon became evident that the latter was a superior product, and in most of the tests the dosage employed was 1 c.c. of ordinary serum and 0·5 c.c. of the concentrated serum.

Here follow a few representative protocols :

(1) 1 *c.c. of Ordinary Serum, or* 1 *c.c. of Concentrated Serum given* 24 *Hours after Inoculation of Rats with* 8 *M.L.D. of* B. pestis. *Five Rats in each Group.*

Ordinary.	Concentrated.	Controls.
3 died between 7th and 9th days	1 died on 14th day	All died in 3–4 days.
2 survivors	4 survivors	No survivors.

(2) 1 *c.c. of Ordinary Serum, or* 0·5 *c.c. of Concentrated Serum given* 24 *Hours before Infection with* B. pestis. *Six Rats in each Group.*

	Ordinary.	Concentrated.	Controls.
1 M.L.D.	Died 11th day	Survived	
2 M.L.D.	,, 6th ,,	,,	
4 M.L.D.	Survived	Died 6th day	All died
8 M.L.D.	,,	,, 14th ,,	within 3 days.
16 M.L.D.	Died 11th day	Survived	
32 M.L.D.	,, 7th ,,	Died 6th day	
	2 survivors	3 survivors	No survivors.

(3) 1 *c.c. of Ordinary Serum at Time of Infection and* 1 *c.c. Next Day.* 0·5 *c.c. of Concentrated Serum at Time of Infection and Repeated Next Day.*

	Ordinary (9 rats).	Concentrated (9 rats).	Controls (6 rats).
1 M.L.D.	2 died ; 1 survived	2 died ; 1 survived	2 died within 4 days.
4 M.L.D.	1 died ; 2 survived	3 died	2 died within 3 days.
16 M.L.D.	2 died ; 1 survived	1 died ; 2 survived	2 died within 3 days.
	4 survivors	3 survivors	No survivors.

It will be seen from these tables that 1 c.c. of ordinary serum and 0·5 c.c. of concentrated serum had approximately the same protective power. This is further borne out by other tests which are not published in detail but gave similar general results.

The numbers of rats saved by a given dose of serum were not exactly in inverse proportion to the dose of *B. pestis*, but it is well known that this cannot be expected with tests employing this organism. We consider, however, that the experiments were on a large enough scale to justify the statement that the concentrated serum, as used, had approximately twice the protective qualitites of the ordinary serum from which it had been prepared. The fully concentrated serum, in other words, was four times as good. This can be considered as satisfactory.

For use the fully concentrated serum (pseudoglobulin fraction) is diluted so that its protective power is approximately three times that of the original serum, the protein content being 12 p.c., which is reasonably and sufficiently low even for intravenous administration. The same dosage is recommended as was formerly advised for the unconcentrated serum, the acute nature of the infection seeming to us to justify this policy.

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MICROSCOPICAL EVIDENCE OF THE EXISTENCE OF SAPROPHYTIC VIRUSES.

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Received for publication February 5th, 1935.

As a biological problem the nature of the " filterable viruses " has assumed recently a different aspect. There is diminishing disagreement with the contention that viruses are small organisms similar to ordinary bacteria, but with some not unexpected physical differences due to their smaller size. At present no definite value can be assigned to their limit of smallness, but there is some reason to expect that the smallest bodies which behave as living organisms approach the size of the largest organic molecule. Perhaps no single objection to the full acceptance of the small organism theory has been so difficult to meet as the lack of experimental evidence of the existence of any non-pathogenic organisms comparable in size with a virus. The number of varieties of non-pathogenic bacteria is immensely greater than those of the known pathogenic varieties ; it is not unreasonable therefore to assume that if viruses really are living things species will exist which are non-pathogenic. The difficulty in proving this assumption is considerable, as hitherto the evidence in favour of the existence of any recognized virus has been mainly and in some cases entirely dependent on the effect produced either in man or animals. Little evidence is available to suggest the existence of what may be termed saprophytic viruses except that Rivers and Tillett (1923) have demonstrated the presence in the testicles of rabbits of a virus that is not normally pathogenic. The object of the present paper is to describe the occurrence in ordinary culture medium of bodies which appear to be examples of saprophytic or non-pathogenic viruses.

In the course of some experiments in which numbers of tubes of serum-broth culture medium were used it was observed that a considerable proportion, taken from stock, showed some slight turbidity or had developed a deposit at the bottom of the tube ; sometimes both changes appeared in the same tube. Apparently these changes have no effect on the value of the medium for ordinary purposes of bacterial cultivation. Thus bovine pleuro-pneumonia, for instance, will grow well in tubes that have such a haze or deposit, and there is no obvious difference in the resulting growth, whether or not before inoculation the medium is hazy or clear. The peculiar morphological characteristics, or perhaps it is more correct to say the peculiar morphological variations, in the cultivated organism occur whether the culture medium is hazy or clear, and the amount of growth in any given time is about the same providing that the tubes have not been kept for too long a time before inoculation.

The culture medium in which the appearances to be described were observed was Hartley's broth to which normal horse- or rabbit-serum had been added

in the proportion of one part serum to five parts of broth. The tubes were kept either on the bench at room temperature or in an incubator at 20° C. There appears to be no marked difference in the changes observed under the two conditions, but tubes kept at 37° C. have not given such satisfactory results. Observations have been made over a period of about five years, and during this time tubes have been examined microscopically every few days. In those tubes in which any suggestion of haziness appeared, the medium or the deposit has been examined microscopically by a visible dark-ground illumination method (Barnard and Elford, 1931). It was soon realized that bodies of uniform size and refractivity were appearing in a small proportion of tubes. Ultra-violet photomicrographs of these bodies showed them to be similar in form to known pathogenic viruses, although other granular bodies which had no characteristic regular form or size also occurred. In some few cases the growth was as uniform and as abundant as would occur with some ordinary bacteria, allowance being made for the smaller size of the bodies we are at present discussing. In most cases a slight haziness developed first, usually in not less than ten days, and later a small chalky deposit formed which was quite distinct from the flocculent deposit so often seen in old serum-broth, and which consists of a heterogeneous mixture of granular bodies among which spheres and particles are found bearing some resemblance to those seen in a culture of bovine pleuro-pneumonia. These spheres and particles are distinct from the subject of this communication and need not be further described here.

Photographs of the deposit in two tubes are shown in Figs. 18 and 19. The intensely white chalky mass (*b*) which consists, apparently, of highly-packed colonies of small organisms can by its characteristic appearance be distinguished readily from the flocculent mass (*a*).

The serum for use in the preparation of serum-broth medium has been obtained as follows : Blood has been collected from normal animals and allowed to stand in the cold room over night. The serum, after being taken off and filtered through a Mandler candle, has been added to the broth without further treatment. The results have been irregular, some batches of medium consisting of about 30 tubes developed nothing suggestive of growth, even when kept for months. Others have shown growth in several, and, exceptionally, in all the tubes in a batch.

Efforts were made to obtain subcultures, but the initial difficulty was to obtain a supply of what may be termed " virus-sterile " culture medium. Sterilization by heat was obviously impossible, although many experiments were carried out to obtain a medium that would, after heating, allow bovine pleuro-pneumonia to grow freely. The medium that showed most promise was prepared by adding to broth heat-coagulated serum digested with trypsin. Although early generations of bovine pleuro-pneumonia grew fairly well in such a medium, successive subcultures became more and more attenuated, and ultimately died out. The only alternative method that suggested itself was to filter the ordinary serum-broth medium through collodion membranes, of such porosity that they would retain the smallest bodies we have been studying, and so obtain what for this purpose might be regarded as virus-free medium.

Experiments have been carried out both with the " gradacol " membranes prepared by Elford, and with acetic acid collodion membranes as originally prepared by Bechhold, with similar results in each case. A typical experiment will indicate the results obtained. Six tubes of horse-serum broth, each containing about 10 c.c., were filtered. The medium appeared to be optically clear, and a microscopic examination of a small quantity from each tube did not disclose the presence of any bodies suggestive of growing organisms. Two of these tubes were inoculated from a stock unfiltered tube known to contain bodies resembling virus particles. The remaining four tubes were uninoculated and kept as controls. All six tubes were kept on the bench at room temperature. After ten days the two inoculated tubes developed a slight haziness, with a barely detectable deposit. After nineteen days one of the control tubes became decidedly hazy, with a little deposit. The remaining three tubes continued to be quite clear and free from deposit after six weeks, when the experiment was regarded as finished. The two inoculated tubes and the control tube which did not remain clear, meantime increased in haziness. In addition to the six filtered tubes described, eleven tubes from the same batch of medium were kept under similar conditions, but were neither filtered nor inoculated. Twenty-two days later seven of these were hazy, with a deposit similar to that in the filtered uninoculated tube and in the two inoculated tubes. The remaining four tubes remained clear to the end of the experiment. Many such experiments have been carried out with different batches of medium, and although the results have been similar to those described, uniformity has not been obtained.

No method of filtration, efficient enough to exclude the organisms observed, consistently yielded a medium in which bovine pleuro-pneumonia grew normally. The latter organism has been used throughout as a test of the growth-promoting power of any medium used, as it so nearly approaches virus size. To overcome the difficulty as far as possible, each batch of tubes was kept for about two months under conditions favourable for growth, and only tubes which remained perfectly clear during this period were retained for attempts at subculture of our saprophytic bodies. Occasionally nearly all the tubes in a batch of medium would remain clear without filtration, and such were regarded as virus-free. Even so, attempts at subcultivation have given irregular results ; in no case have regular subcultures of our virus bodies been carried on for more than four generations. The amount of visible growth has been less in each subculture until finally no growth has occurred in suitably prepared or selected tubes. The justification for the assumption that the bodies appearing in the tubes are small living organisms which multiply by binary fission, is dependent on their microscopical appearance. This evidence is presented in the accompanying illustrations. It will be appreciated that no reproduction, however excellent, can give the sharpness of detail of the original negatives.

The photomicrographs are all taken by a dark-ground illumination method using ultra-violet light. The wave-length used is 275 mμ, and the effective numerical aperture of the quartz monochromatic objective used at that wave-length is 2·30. The optical system is therefore adequate for the resolution of the smallest of the bodies shown. In all cases the material observed was

taken direct from the culture-tube, and a preparation was made between quartz-slide and cover-glass without any process of fixing or staining. Photographs of *B. megatherium*, *B. bronchisepticus* and vaccinia taken under these same conditions are included to facilitate comparisons. The magnification throughout is × 3200.

Fig. 1 shows *B. megatherium*, Fig. 2 *B. bronchisepticus*, and Fig. 3 vaccinia from rabbit testicle.

Figs. 11 to 16 are photographs of bodies, presumably organisms, occurring in tubes of serum-broth media as described above. It is suggested that three varieties of organism are shown here, but any differentiation is entirely dependent on their apparent size and form.

Figs. 5 to 8 represent the smallest bodies of the series and it is clear that the organisms occur mainly in pairs, and that subdivision is taking place. In diameter these are about the same as vaccinia, from 0·15 to 0·17μ.

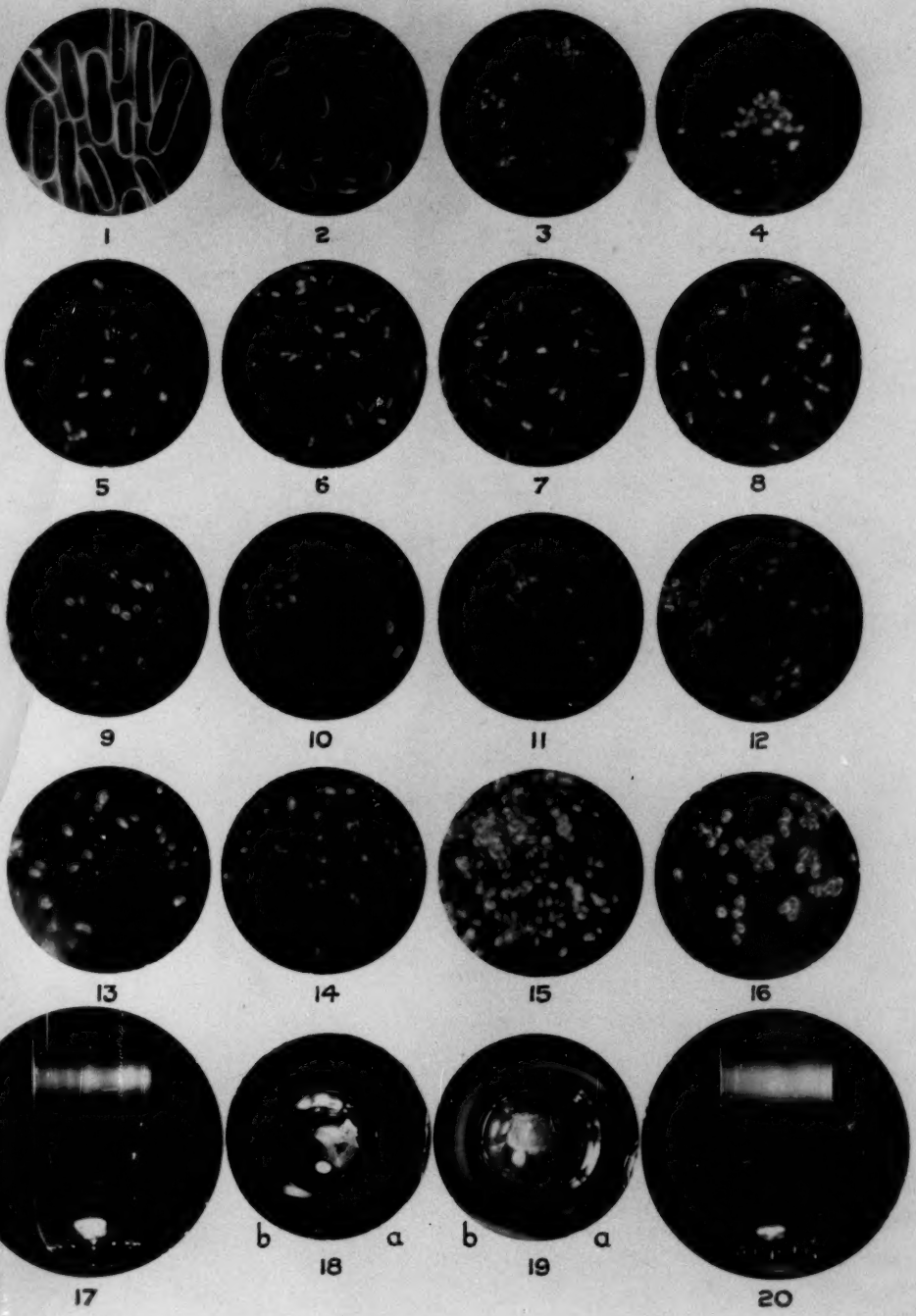
The bodies represented in Figs. 9 to 12 are somewhat larger than those shown in Fig. 5, but appear to be variable in size; some organisms are relatively large, but much smaller forms are to be seen. In Figs. 13 and 14 they are probably the same variety and show the same characteristics. Both these figures are photographs of the edge of a mass of organisms, which, it must be assumed, is a colony growing in the fluid and probably, therefore, a pure culture.

Fig. 15 is again from a small colony-like mass, the edge of which has spread out under the pressure of the cover-glass, and shows the individual organisms. Fig. 16 is probably the same organism as Fig. 15, but from another batch of medium.

Figs. 17 and 20 are photographs of the lower portion of two tubes of serum-broth from the same batch. Fig. 17 is of a tube in which no detectable growth occurred, the medium remained clear. Fig. 20 is of a tube in which growth developed. It was often difficult to determine in daylight whether a tube was becoming hazy, particularly in its early stages. The method was adopted, therefore, of illuminating the tubes with a narrow slit-image from an electric arc, which showed up as a bright band in the medium as seen in the photographs. If white light is used, considerable scattering of the blue component of the light causes the beam to spread out and appear broader. This tendency was overcome by using an orange-yellow colour-screen, and the photographs were taken by this method. The difference in width and intensity of the beams indicates the difference in the amount of particulate matter in the two tubes. The white patches shown at the bottom of the tubes are due to internal reflection; they are not due to sediment.

SUMMARY AND CONCLUSIONS.

Refined microscopical examination of the deposits which sometimes occur in medium containing horse- or rabbit-serum reveals the presence of minute bodies which, in size and constancy of size, in shape and in constancy and regularity of shape, and also in general optical properties, are in no way different from acknowledged pathogenic viruses. The bodies increase in numbers, and photographs show them in process of fission. They have been cultivated



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to the fourth subculture. It is concluded that these bodies are of virus nature.

This demonstration that viruses do occur, which are saprophytic in habit and which multiply in the absence of tissue-cells, answers in itself certain theoretical objections to the view that viruses are living organisms.

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STUDIES ON THE B VIRUS. IV: HISTOPATHOLOGY OF THE EXPERIMENTAL DISEASE IN *RHESUS* MONKEYS* AND RABBITS.

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Received for publication February 20th, 1935.

THE B Virus was isolated from a human case of ascending myelitis which followed the bite of an apparently normal monkey. In addition to the pathological changes in the nervous system, striking features of the case were vesiculo-pustular lesions on the fingers at the sites of the monkey bites, and areas of necrosis in the regional lymph-nodes, adrenals and spleen. The same virus was obtained from both the brain and cord and from the spleen (Sabin and Wright, 1934). The experimental disease produced by this virus in rabbits, guinea-pigs and *rhesus* monkeys has been previously described (Sabin, 1934*a*, *b*, *c*). The virus possesses certain features in common with the viruses of herpes simplex and pseudorabies, and while it was found to be immunologically distinct there was some evidence suggesting an immunological group relationship among the three. Of the three viruses only the B Virus and that of pseudorabies are pathogenic for *rhesus* monkeys. From evidence presented in a preceding communication (Sabin, 1934*c*) it appears highly probable that the B Virus is indigenous to *rhesus* monkeys.

Although some of the lesions produced by the virus in the rabbit have already been described (Sabin and Wright, 1934), no systematic study was made; evaluation of the changes in the central nervous system was particularly

* *Macaca mulatta.*

difficult on account of the high incidence among rabbits in America of lesions due to *Encephalitozoon cuniculi*.

The present article describes the histological features of the disease in monkeys, and amplifies the details already published of those in the rabbit.

GENERAL FEATURES OF THE LESIONS.

A primary attack of the virus on cells derived from all embryonic layers leads to their speedy destruction and widespread acute necrosis, yet in the case of certain tissues, not before the cells have passed through a definite, if brief, proliferative stage. The customary acute and later subacute inflammatory reactions rapidly supervene, and reparative processes are initiated. Prominent among the lesions is necrosis of the blood-vessels; not infrequently thrombosis occurs and doubtless contributes to the general destruction.

These essential features are observed alike in the monkey and the rabbit; the chief difference between the process in the two species resides in the slighter tendency in the monkey towards invasion of the central nervous system from a peripheral focus of infection, and the more purely mesodermal nature of the nervous lesions.

In the pre-necrotic phase acidophilic nuclear degeneration produces inclusions probably indistinguishable from those of herpes and of pseudorabies; their formation accompanies fragmentation of the nucleolus and margination of the basophilic chromatin. The various appearances in Zenker-fixed tissues stained with phloxine-methylene blue may be summarized as follows :

(1) Coarse, deep red granules commonly aggregated near the centre of the nucleus to form an ovoid or irregular clump separated by a clear space from the nuclear membrane (Fig. 9*b*). Alternatively, the granules may be loosely scattered throughout the nucleus or tightly packed to the limits of a distended and bursting nucleus.

(2) Smaller or larger irregular masses of not clearly granular deep red material (Fig. 8*a*).

(3) Fine or coarse weakly oxyphilic granules arranged as in (1) (Fig. 8*b*). When very fine and very tightly packed, as is more especially the case in a proportion of nerve-cells in this disease and in pseudorabies, the nucleus under a moderately low power appears diffusely pink. In some cells also the most perfect fixation and careful staining may not prevent the inclusion material from assuming a lilac or purplish tint.* In consequence, the significance of these changes may not at once be obvious to the observer seeking a " typical " inclusion of the commoner varieties.

(4) Multiple oxyphilic spherules. Rarely these bodies have a central paler area exactly like those in Borna disease, equine encephalomyelitis and poliomyelitis.

* This basophilic staining of certain inclusions is a point requiring further study. The proportion of basophilic inclusions and the degree of their basophilia seems to depend on several factors, among which are the nature of the fixative, the stain employed, and the species of animal (see Hurst, 1933). Basophilia is not characteristic only of inclusions at an early stage of development, as the account of Nicolau, Kopciowska and Mathis (1934) implies. As stated, the description above is of sections coloured with phloxine-methylene blue after fixation in Zenker with acetic acid (or in sublimate-formol). With iron-alum hæmatoxylin and eosin many stain purplish or blackish; with methyl blue-eosin after sublimate fixation the great majority are frankly blue.

(5) Combinations of the above forms may occur.

Further progress towards destruction of affected cells is sometimes signalled by rupture of the nuclear membrane, but usually by shrinkage and pyknosis of the nucleus and increasing acidophilia of the cytoplasm. In the series of cases to be described inclusions were present in all the tissues affected by the disease in both rabbits and monkeys ; the one exception was that of a monkey dying of a secondary penumonia on the fifteenth day, when the primary infection had obviously passed its zenith.

LESIONS IN THE MONKEY.

The following animals were examined histologically ; detailed clinical findings have already been reported (Sabin, 1934*c*).

No.	Route of inoculation.	Course of disease.
3.	Intracutaneous and intraperitoneal	Biopsy of skin-lesions 4th day. Recovery. Autopsy 7 months later.
4.	Intracerebral and intraperitoneal	Nervous symptoms 2nd day. Death and autopsy 3rd day.
6.	Intracerebral and intraperitoneal	Nervous symptoms 4th day. Death and autopsy 5th day.
7.	Intracerebral and intracutaneous	Nervous symptoms 2nd day. Killed and autopsy performed 3rd day.
8.	Intravenous and intracutaneous	Biopsy of skin-lesions 3rd day. Killed and autopsy performed 15th day.
9.	Intracerebral, intraperitoneal and intracutaneous	Nervous symptoms 3rd day. Death and autopsy 4th day.
15.	Intracerebral and intraperitoneal	Nervous symptoms 6th day. Killed and autopsy performed 8th day.

Nervous System.

(a) *Lesions in M. 4, 6, 7, 9 and 15.*

The lesion constantly present in intracerebrally inoculated animals is an intense meningitis most marked nearer the site of inoculation, but affecting also the opposite hemisphere, the brain-stem and cerebellum, and extending for a variable distance down the cord, even as far as the lumbar region. Somewhat resembling that in cerebral vaccinia (Levaditi, Nicolau and Sanchis Bayarri, 1927 ; Hurst and Fairbrother, 1930 ; Rivers, Sprunt and Berry, 1933 ; Pette and Környey, 1933), in the present disease the exudate is far less cellular and accompanied by more acute destruction of tissue. Where the process is most severe, the meshes of the pia arachnoid are distended with abundant fibrinous exudate containing relatively few polymorphonuclear leucocytes, later admixed with lymphocytes and large mononuclears ; numerous fine chromatic granules represent karyorrhectic nuclei, many, no doubt, of infiltrating polymorphs, but also of cells proper to the meninges (Fig. 4). By the eighth day it is obvious that replacement of the original polymorphonuclear infiltrate by mononuclears is proceeding. Where in less affected areas the fibrinous element of the exudate is much slighter, the underlying cellular lesions are more clearly appreciated ; inclusions precede necrosis in every type of meningeal cell, abundant polymorphs appear only to suffer karyorrhexis, fresh polymorphs arrive, and proliferation of surviving meningeal elements begins.

The walls of the meningeal vessels participate in this process. The adventitia is almost always involved in some degree and is often wholly necrotic and heavily infiltrated with polymorphs ; in less damaged vessels marked cellular proliferation occurs alone or side by side with some necrosis. Nuclear inclusions may be abundant. Not infrequently the muscular coat is necrotic through its whole thickness over part or all of the circumference of the vessel ; still more often,

as seen admirably in tangential section, individual muscle-cells are necrotic among many survivors. Inclusions are seen uncommonly; apparently when affected by the virus the muscle nucleus passes into the pyknotic stage more rapidly than do those of connective-tissue and some other cells, with the result that the number of inclusions present at one time is small. Polymorphonuclear infiltration obtains also in this coat. The lining endothelium seldom exhibits inclusions, though in other situations (*e.g.* locally after intratesticular inoculation in the rabbit) almost every cell may be involved, and desquamated elements bearing nuclear inclusions may be detected in the vascular lumen.

In other parts of the cerebrum changes are ordinarily confined to an extension of the meningeal lesion downwards along the perivascular sheaths of the penetrating vessels as far as the first or second cortical layer, with, in places, scanty polymorphonuclear infiltration in the first cortical layer. Only one animal (see below) shows more definite nervous involvement at a distance from the site of inoculation.

(*b*) *Additional Lesions in M. 15.*

The actual site of inoculation was sectioned only in this animal. The changes here are qualitatively and quantitatively far different from those due to simple trauma; in part they can be attributed to a direct attack of the virus, and in part to vascular thrombosis, itself an indirect result of virus action. Widely-spread, hæmorrhagic necrosis coincides with thrombosis of many of the medium-sized and small vessels, complete necrosis of their walls and a tissue infiltrate composed of pyknotic and karyorrhectic polymorphs.

Slightly removed from the central area, the highly œdematous tissues are incompletely necrotic, with survival of some of the interstitial cells; considerable polymorphonuclear infiltration is represented chiefly by pyknotic cells with only a small proportion of well-preserved leucocytes. The vessels exhibit pronounced activity on the part of endothelium and adventitia with active capillary budding; perivascular infiltration with lymphocytes and polymorphs in about equal numbers is far from marked, and sometimes absent, most of the cells in the Virchow-Robin spaces being proliferated adventitial elements. In the surrounding tissues microglial cells, degenerate and healthy polymorphs, and a few lymphocytes are accumulated. The nerve-cells are represented by structureless eosinophilic masses. Glial nuclei are pale and greatly swollen, usually without oxychromatic material, but sometimes showing typical inclusions (Fig. 9*b*). The microglia is proliferating and often displays mitotic figures.

Still further away most nerve-cells survive, though many are swollen and vacuolated and possess no Nissl bodies, and some few are necrotic. Considerable diffuse microglial proliferation, some polymorphonuclear invasion, and reactive swelling of the glia are evident in the interstitium. The tissue infiltrate is often accentuated around the vessels, which again exhibit but few inflammatory cells in the Virchow-Robin spaces and much swelling of adventitial and, to a less extent, of intimal elements (Fig. 5). From endothelial swelling all the capillaries stand out boldly. In the cortical zone immediately beneath the pia focal glial necroses are associated with polymorphonuclear infiltration and microglial overgrowth.

In the general cortex of this animal the subpial glial-cells are affected with some frequency; many are necrotic, their position being marked by leucocytic invasion and microglial proliferation, others enlarged and bearing nuclear inclusions. Numerous small areas of the cortex contain a few necrotic nerve-cells, while neighbouring neurons and glial-cells manifest specific alterations; slight leucocytic infiltration and absence of microglial activity bespeak the recent establishment of these foci. Sometimes such areas are intimately related to a vessel entering the cortex from an affected part of the pia arachnoid, and perhaps infiltrated over a longer stretch than usual. At other sites, especially in the contralateral hemisphere, the overlying membranes are free from change, suggesting spread of the virus by a route other than the meningeal. Similar appearances are present in the pons in the nucleus of one fifth nerve.

In the dorsal spinal ganglia of this monkey, occasional nerve-cells (from one to three in a section) are acutely necrotic, usually without signs of cellular reaction. Here and there early multiplication of the capsule-cells is apparent, or rarely neuronophagia of the dead cell. The localization of these lesions in the dorsal region, and the absence of nervous changes between this level and the but slightly affected pons, suggest that here the neuronal destruction reflects the ascent of virus by the peripheral nerves from the peritoneum rather than a descent from higher regions.

(c) *Nervous Lesions after Peripheral Inoculation of Virus.*

The nervous system of the fifteen-day monkey inoculated intravenously and intracutaneously (M. 8) shows very scanty lesions. In the medulla a few microglial nodules exist, and in the posterior horns of a dorsal segment of the cord there may be slight excess of microglia. At this stage no specific lesions can be detected. Unfortunately, the spinal ganglia were not examined.

To summarize, therefore, the histological findings in the nervous system are in accord with the clinical data on which comment has already been passed (Sabin, 1934*c*). While peripheral inoculation unaccompanied by intracerebral injection of virus is probably associated with minimal lesions at a corresponding level of the spinal cord, and perhaps in the medulla, in the monkey systemic infection does not lead to the striking and rapidly fatal invasion of the nervous system characteristic in the rabbit ; the course of the resulting malady may be relatively benign. Intracerebral inoculation is needed to evoke a fatal meningo-encephalitis in which the meningeal element predominates.

Lesions in Other Organs.

In the abdomen following intraperitoneal inoculation (M. 4, 6, 9, 15) the *intestines*, *omentum* and *mesentery* adhere in a matted complex of which the individual constituents are with difficulty separated. In M. 9 and 15 large areas of necrosis occur in the submucosal lymphoid tissue of the gut, beneath the serosa, in the omental and mesenteric adipose and areolar tissues, and in the mesenteric lymph-nodes. As in the brain, the walls of the vessels are severely affected, and vascular thrombosis is not infrequent.

The centres of the necrotic areas often consist of nothing but degenerate polymorphs surrounded by a structureless necrotic zone. Peripheral to this is a zone of epithelioid cells in which nuclear inclusions may be abundant, and in which, especially in the lymph-nodes, many enormous multinucleated giant-cells are present. Advancing necrosis has involved many of the latter, which can still be recognized in outline, and often the living giant-cells exhibit inclusions in each one of their numerous nuclei (Figs. 3, 10). (The monkeys presented no signs of tuberculosis, and careful search revealed no bacilli ; moreover, we have never observed giant-cells approaching these in size in any case of monkey tuberculosis.) Further from the centre numerous lymphocytes and plasmocytoid cells, perhaps some polymorphs and eosinophils, and later a few typical plasma-cells, fibroblasts and budding endothelial cells mingle with epithelioid cells and smaller giant-cells. Here and there necrotic lymphocytes occur, but in none can inclusions be detected.

In one mesenteric lymph-node of M. 15 a different picture is seen. No specific changes are present, and the node is converted *in toto* into a structureless mass in which phantom outlines of blood-vessels and germinal centres are barely distinguishable. Presumably vascular blockage, and not a direct attack of the virus, is the factor responsible for this destruction.

Liver.—Monkeys 6, 8, 9 and 15 exhibit focal necroses throughout the substance of the organ. The foci, consisting at first of two or three cells, and later including an appreciable fraction of the hepatic lobule, occupy indiscriminately periportal, midzonal and central positions. From an early stage both liver- and Kupffer-cells contain nuclear inclusions ; it is not possible to state which is first affected. The central parts of the older foci may be completely necrotic, or may show a few surviving or invading endothelial cells ; the sinusoids are often thrombosed and small hæmorrhages are frequent. When enclosed within a large focus the central vein may or may not be thrombosed ; its wall may be partly or wholly necrotic. The portal tracts are markedly infiltrated with mononuclears, or with polymorphonuclears when a necrotic focus is contiguous ; inclusions are seen in connective-tissue cells and in the lining cells of certain bile-ducts. Endothelial proliferation is to be noted in some branches of the portal vein.

In addition, in M. 6, 9 and 15 (inoculated intraperitoneally) changes are present immediately beneath the capsule, itself necrotic and covered with a thick layer of fibrin. In M. 6 the early stages of the process are evident chiefly in the capsule, where nuclear inclusions, necrosis and leucocytic exudation obtain ; only in places are the subjacent hepatic and Kupffer-cells affected. In M. 15 a continuous necrotic band some thirty to eighty liver-cells deep exists under the capsule (Fig. 1).

Recently dead liver-cells, and inclusions in the neighbouring liver- and Kupffer-cells, are still numerous in M. 15 on the eighth day (Fig. 8). On the fifteenth day (M. 8) inclusions are absent, the initial polymorphonuclear exudate has been replaced by mononuclears, reparative processes are active, and much of the necrotic *débris* has disappeared.

Spleen.—As in the case of the liver, after intraperitoneal injection the capsule is covered with fibrin, its fibro-muscular tissue partly or wholly necrotic and infiltrated with inflammatory cells. In cases of longer duration (M. 6, 9 and 15) necrotic changes are present in the splenic tissue immediately subjacent.

Necrosis in the deeper parts of the organ begins at the boundaries of the Malpighian bodies and spreads thence into the pulp, where in M. 15 it involves in all perhaps a quarter of that tissue (Fig. 2). Here, in places, all structure is lost, the vessels and fibro-muscular septa sharing in the general destruction; comminuted nuclei pepper the tissues, and much fibrin has been formed. Around these areas marked polymorphonuclear infiltration, endothelial proliferation and activity of vascular adventitia and intima are associated with abundant specific alterations in the nuclei of a great variety of cells. In small arteries and fibro-muscular septa, the muscle-cells are commonly necrotic with pyknosis of their nuclei, while the more resistant connective-tissue elements survive for a time, bearing nuclear inclusions. More normal parts of the pulp are greatly congested, and their sinuses contain an excess of polymorphonuclears.

Although in the Malpighian bodies isolated dead or pyknotic cells and occasional leucocytes may be present, the integrity of the corpuscles as a whole is almost always respected, and massive necrosis arrested at their confines. This picture contrasts with that in the vaccinial monkey, where leucocytic replacement of the cells of the Malpighian body is very conspicuous, and necrosis of the whole nodule may sometimes supervene (Hurst and Fairbrother, 1930).

In M. 8 surprising progress has been made in the resorption of the destroyed tissues.

Adrenals.—In each of the four monkeys dying on or after the fourth day (M. 6, 8, 9 and 15) these structures are affected. The lesions progress from necrosis of individual cortical cells, often betrayed by a gap occupied by polymorphs in one of the cell columns, to large and confluent foci of destruction involving, in M. 8 and 15, three-fourths of the cortex and the whole of the medulla. Vascular changes resemble those elsewhere. A large proportion of surviving glandular and proliferating endothelial cells bear inclusions, which at an early stage may also be found in sympathetic nerve-cells of the medulla. The sinuses of relatively intact areas are greatly injected. In M. 8 excessively pale and foamy glandular cells surround the necrotic tracts and occur in irregular foci in the cortex; whether these, as their situation suggests, correspond to cells previously containing inclusions and not irreparably damaged by the virus can only be a subject for speculation.

Ovary.—Monkeys 8 and 15 show extensive necrosis of the stroma and germinal apparatus, with the usual vascular and infiltrative concomitants. In the latter case inclusions are present in every type of cell, including ova and follicular epithelium at all stages of development of the germinal vesicle, germinal epithelium, and epithelium of the fimbriæ of the Fallopian tube.

The *kidney* is involved only in the monkey inoculated intravenously (M. 8). Certain of the arcuate and interlobular arteries exhibit the reparative stage of a vascular process in no way different from that in other organs; in a few places extension along an afferent arteriole towards a glomerulus is evident, or an inflammatory focus in the interstitial tissues. Some few glomeruli are the seat of epithelial proliferation, but for the most part the parenchyma is normal.

Again, only in this monkey do the *lungs* present pathological changes, in the form of severe confluent broncho-pneumonia. At this stage it cannot be decided whether or not the lesion developed on the basis of preceding changes of specific nature; the picture in no way resembles that of a virus pneumonia.

Lesions in the *skin* of intracutaneously inoculated animals furnish an excellent demonstration of the proliferative stimulus of the infective agent at an early stage. When vesicles are developing, the loosened epithelial masses are packed with nuclei much smaller and in much closer apposition than normally, while in the hair-follicles the same process is evident without disorganization of structure. The presence of inclusions in these nuclei serves to indicate the relationship of the virus to the process of division (Fig. 9*a*).

The earliest lesions available for study are already, on the third day (M. 7 and 8), in the stage of vesiculation; the separating epithelial cells, together with those of the surrounding epidermis, all contain, or appear to have contained, nuclear inclusions. All the deeper epithelial layers soon become detached, polymorphs migrate from the underlying tissues, and, when the horny

layer gives way, the floor of the " ulcer " is composed of necrotic corium. Necrosis and inflammation in the latter occur independently of secondary infection and present all the essential features previously described.

As has been stated elsewhere, in the presence of the nervous disease engendered by intracerebral inoculation, the evolution of the skin-lesion appears to be partially inhibited, and little difference is apparent between the picture on the third day following simple intracutaneous inoculation, and that at a much later period, when intracerebral has been combind with peripheral inoculation.

On the fifteenth day (M. 8) regeneration of epithelium is prominent, while in the corium the dead tissue has been partly removed and the tissue infiltrate is composed mainly of lymphocytes, plasmacytoid cells and small giant-cells. In this animal, it will be recalled, intravenous inoculation had resulted in eruptive lesions on skin and mucous membranes; appearances in the healing ulcers on the tongue and lips were substantially the same as those in the skin.

Sequelæ of the acute attack.—Considerable interest attaches to the pathological findings in Monkey 3, surviving seven months after intraperitoneal injection of a large dose of virus. Autopsy revealed the sequelæ of a severe peritonitis; the liver and spleen were adherent to the diaphragm and intestines. Sections of the organs show little beyond the chronic changes (fibrosis) in their capsules; no traces of parenchymatous necrosis or of subsequent scar-formation exist. There seems no reason to doubt that in the acute stages of the disease this animal suffered in the same manner as the preceding monkeys; the present observation points, therefore, to the possibility of complete reparation of extensive parenchymatous damage in the viscera. In this respect it would appear that the visceral lesions conform more closely to those produced by poisons of various kinds than to those directly conditiond by bacteria.

In short, it seems likely that in the monkey the B Virus can produce destructive lesions in any and every tissue and organ. Under common experimental conditions, however, certain organs are more subject to attack; these are the liver, spleen, adrenal, ovary and lymph-nodes. In spite of the severity of these visceral lesions, the slight tendency towards invasion of the central nervous system does not preclude the possibility of recovery, and the limited evidence available suggests that restitution of the normal structure may be remarkably complete.

LESIONS IN THE RABBIT.

Some attention has been paid elsewhere to the general character of the lesions in this animal (Sabin and Wright, 1934); the present description provides further data, particularly of the sequence of events leading to invasion of the central nervous system, and of the distribution of changes following inoculation by various routes.

Intracerebral Inoculation.

One animal, killed when showing advanced symptoms 52 hours after inoculation, exhibits alterations in the nervous system essentially similar to those in pseudorabies (Hurst, 1933), though rather more advanced and widespread. Compared with lesions in the monkey, meningitis appears a less, and nerve-cell involvement a relatively more, important factor.

Although present where meningitis is most intense, necrotic vascular changes are far less frequent than in the monkey and seldom involve the muscular coat; necrosis of various cells of the meninges is also less obvious. Inclusions are of widespread occurrence in connective tissue, mesothelial cells, etc., and at an early stage are associated with scanty lymphocytic infiltration and perhaps with adventitial proliferation in local vessels. Where necrosis occurs,

abundant polymorphs exude and suffer karyorrhexis. Fibrinous exudate is minimal. Such meningitis is patchy over the cerebral hemispheres, marked over the cerebellum, and present even in the lower cord in the angles around the posterior roots. Although the shorter duration of the disease in the rabbit doubtless accounts for the less severe meningeal changes, this in itself probably implies more severe parenchymatous involvement leading to earlier nervous symptoms and death.

Mononuclear sheaths surround a few of the cortical vessels and those beneath the ependyma of the third ventricle. In other places slight polymorphonuclear cuffing is associated with the presence of nuclear inclusions in the adventitial cells. In the granular layer of the cerebellum polymorphonuclear perivascular infiltration is present apparently without advential inclusions.

At the site of inoculation massive necrosis is not observed, though abundant inclusions exist in the surrounding glia. In many areas subpial and subependymal glial-cells bear inclusions to a much greater depth from the surface than in pseudorabies. Throughout the ventricular system patches of ependymal cells are similarly affected, including those of the central canal of the spinal cord as far as the lumbar region where the underlying glia also participates.

Superficially situated nerve-cells often contain inclusions, and in some regions (*e. g.* in the basal ganglia) deeper nerve-cells are implicated with great frequency. Rare inclusions are present in the pyramidal cell band of the cornu Ammonis, and large numbers in the neurons and glial-cells of the rhinencephalic cortex, where necrosis is imminent. Nerve-cells immediately beneath the ependyma of the fourth ventricle are involved, but not those of the cerebellum or spinal cord. The cervical and lumbar intervertebral ganglia are normal.

Intramuscular Inoculation.

Four rabbits were inoculated intramuscularly in the calf and killed on the second, fourth, sixth and eighth days respectively.

At the forty-eighth hour necrotic muscle-fibres, often partly or wholly buried in an exudate of karyorrhectic leucocytes, are already evident; adjacent fibrous septa are widely infiltrated with various mononuclear elements. Inclusions are not seen in muscle, but are of limited occurrence in connective-tissue cells of various types. Inclusion formation and necrosis is beginning in the walls of blood-vessels and the connective-tissue sheaths of nerve-bundles; the nerve-fibres seem as yet uninvolved, though inflammation of the sheath continues for some distance up the innervating trunk. A regional lymph-node exhibits some peripheral leucocytic infiltration without obvious specific changes. The intervertebral ganglia, spinal cord and general viscera are normal.

On the fourth day necrosis of muscle, vessel-walls and nerve-sheaths is more advanced. In a few muscle-fibres otherwise apparently normal considerable multiplication of sarcolemmal nuclei may be noted. Inclusions are rarely detected in affected muscle nuclei, which rapidly become pyknotic; in other cells, including macrophages phagocyting the dead muscle, they are numerous in the immediate vicinity of necrotic zones. Endothelial budding and fibroblastic proliferation accompany exudation of abundant polymorphs, lymphocytes and many macrophages. Interstitial infiltration of some nerve-bundles follows upon destruction of their fibrous sheaths. A regional lymph-node is the seat, near its periphery, of specific changes in the form of marked multiplication of endothelial and reticular cells bearing inclusions; significant necrosis has not yet occurred, but slight fibrinous exudate and a few karyorrhectic leucocytes are present. The central nervous system and general viscera are normal.

At 6 days paralysis of the posterior extremities has recently developed. At the site of inoculation wide tracts of almost structureless tissue result from the massive necrosis of muscle, interstitial tissue, vessels and exudate; many small extravasations of blood are seen. Surrounding areas suffer from an extension of the process described above, and numerous inclusions bear witness to the continuing activity of the infective agent. At the same time attempts at repair are active. Interstitial neuritis affects the highest portion of the sciatic nerve examined, and is most intense in one part of the trunk, corresponding no doubt to the fibres supplying the inoculated muscles (Fig. 6). Some œdema and a preponderantly mononuclear exudate separate the nerve-fibres; in certain foci cellular necrosis has evoked a polymorphonuclear reaction. Inclusions exist in connective-tissue cells and in the nuclei of the sheath of Schwann, and moderately advanced

changes obtain in the cord and ganglia (see below). The lung, heart, spleen, adrenal, liver, kidney and ovary are free from necrotic foci.

On the eighth day, with the animal *in extremis*, but few differences are noted in the muscle, though polymorphonuclear exudation is diminishing. Inclusions are few and far between, being seen chiefly in occasional greatly distended nuclei apparently about to burst. The condition of the sciatic nerve resembles that in the preceding case; inclusions are still seen in some Schwann-cells, etc. Advanced lesions are found in the lower segments of the spinal cord, and earlier lesions above. One adrenal displays a large necrotic focus at the junction of cortex and medulla, and the ovary early focal necrosis of the interstitial cells. No necrosis is apparent in other viscera.

Intradermal Inoculation.

The nervous system was studied in a number of cases following intradermal inoculation, as well as in the preceding series of intramuscularly injected rabbits.

In recently affected spinal ganglia the primary lesion does not appear to be invariably the same. Usually degeneration first strikes the nerve-cells, which pass through a series of changes extremely like, if not identical with, those in pseudorabies (*q.v.*). Acidophilic nuclear degeneration leading to the formation of intranuclear inclusions precedes necrosis of the neuron; meanwhile the capsule-cells proliferate and contain inclusions. Often a fair sprinkling of the neurons may be thus affected in the absence of all cellular infiltration. In one instance, however, at the level corresponding to the site of inoculation, the histological picture suggests that occasionally a ganglion may be involved by direct extension of the ascending neuritic process from the posterior root, when at an early stage interstitial inflammation is out of all proportion to the number of nerve-cells destroyed. Even in the former circumstances, the longer duration of life after virus first reaches the central nervous system permits the development of massive inflammatory changes and interstitial destruction to an extent never witnessed in pseudorabies in the rabbit, and exceeding that commonly present in pseudorabies in the guinea-pig (Fig. 7). The earliest cellular reaction observed is often largely mononuclear, but where many neurons are necrotic they disappear beneath a horde of polymorphs, while sometimes enormous numbers of leucocytes infiltrate the whole substance of the ganglion. They are, however, rapidly replaced by proliferated capsule-cells and other mononuclears forming nodules prominent in the general infiltrate. Later, in an interstitium densely infiltrated with lymphocytes, a few polymorphs, many macrophages, etc., the endothelium of the small vessels while rarely showing specific changes becomes markedly swollen. At death the ganglia corresponding to the inoculated muscle may possess no single living nerve-cell, and but few recognizable remains, while at higher levels (cervical) the earlier stages of involvement may be found.

In the spinal cord the tip of one posterior horn may show earliest affection, quickly followed by the adjacent white matter, the deeper parts of the posterior horn, and the region of the central canal; the anterior horn lags only slightly behind and the opposite side is soon attacked. In other instances the anterior horns appear to be earliest involved (Fig. 11). Typical changes are present in nerve, glial and ependymal cells of the cord and in Schwann-cells of the anterior and posterior nerve-roots. At first there may be slight or moderate leucocytic infiltration, and some microglial proliferation, especially at the point of entry of the nerve-roots. Soon, however, the polymorphs suffer karyorrhexis and the microglia is itself attacked by the virus, with the consequence that relatively little cellular increase is obvious even after several days. In the cord neuronophagia never attains the prominence it does in, say, poliomyelitis; often, indeed, no attempt at removal of dead neurons is made. When death occurs the first affected segments of the spinal cord are so diffluent that only with the greatest difficulty can material for study be secured. Almost every surviving cell of the grey and the white matter, except perhaps for a few microglial cells in process of conversion into granular corpuscles, contains inclusions; the majority are necrotic. At higher and at lower levels the intensity of the lesions diminishes.

From an early, but not the very earliest stage, the veins and to a slighter degree the arteries of the grey and white matter exhibit mononuclear cuffing; adventitial and endothelial activity are pronounced. Specific changes and polymorphonuclear reaction supervene in only a very small minority of vessels. The foregoing remarks apply equally to the small vessels of the nerve-roots.

The meninges, too, at first in relation to one posterior horn and then over a wider area, but still chiefly posteriorly, are infiltrated with mononuclears; later on a very few inclusions but no necrotic cells are present. In the sparsity of specific lesions the pial elements in the later stages of the malady contrast sharply with the immediately subjacent glia, and those of the vessel-walls in the cord with closely adjacent glial-cells. The observation seems of some importance as implying one of two possibilities; either that virus is delivered *into* the nervous tissues in much greater concentration than into the membranes, as for example by passing along "insulated" axis cylinders, or that given equal opportunities of infection the nervous structures pass much more rapidly through the various stages than do the mesodermal. Now the interval between the onset of nervous symptoms and death is commonly two days or so, and experience with the brain following intracerebral inoculation shows that after a similar period mesodermal lesions may be well advanced. Were the latter alternative the correct one, therefore, we should still expect more abundant specific lesions in the meninges during the terminal stages, at levels corresponding to the site of inoculation and consequently infected for a minimum period of two days. Their paucity points to the former factor as the main determining one. In the cord, then, the alterations in both vessels and meninges can probably be construed as largely in the nature of a reaction to the very severe nervous damage, rather than as specific lesions representing a direct attack of the virus on these structures.

In the days following the initial paralysis, lesions spread more or less slowly to higher levels. Infection of the ependyma of the whole central canal is usual, and may extend to that of the fourth ventricle. At death early changes are present in the cervical cord, and foci of nerve and glial-cell involvement with vascular reaction in the brain-stem; in the latter region, however, lesions are far from conspicuous, while the cerebrum may escape visible damage.

Intratesticular Inoculation.

The following are the findings in a rabbit inoculated into both testes.

One testis surgically removed 66 hours after inoculation is affected severely in its central part, while its two poles are still free from obvious abnormality. With a low magnification enormous dilatation of the vessels combines with confluent interstitial hæmorrhage in arresting attention. With a higher power, considerable cellular necrosis, the customary karyorrhectic polymorphonuclear infiltration, and nuclear inclusions in almost every surviving cell in the interstitium are an index of the severity of the virus attack. Some vessels are already necrotic through to their endothelial lining, which may contain inclusions; others show earlier involvement of their walls with appearances already detailed. Some of the seminiferous tubules are necrotic in whole or in part, but considering the state of the surrounding interstitial tissue it is often surprising how nearly normal others appear, and how mitosis continues for a time in apparently normal manner. No inclusions are seen with certainty in the spermatogonia, though they are at times present in the nuclei of the basement membrane.

On the fifth day, before nervous symptoms were due to develop, the rabbit was killed. In the remaining testis the destructive lesion has increased greatly in extent. Over a great part of the body of the organ, an intensely hæmorrhagic necrosis involves all structures save a few of the vessels, occasional seminiferous tubules, and parts of the epithelium of the *rete testis*. Surviving vessels exhibit considerable adventitial activity, but the muscular coat is necrotic and the elastic fibres fragmented; the endothelium, like many of the adventitial cells, contains inclusions. Interstitial degeneration, inflammation and hæmorrhage extend into the epididymis, where many epithelial cells display inclusions.

This case is notable for the numerous secondary necrotic foci in the liver and adrenal. In the liver the distribution in the lobules is mainly periportal and midzonal, and rarely central. In general resembling those in the monkey, the present lesions show much more polymorphonuclear exudate and multiplication of Kupffer-cells. The still living, but inclusion-bearing hepatic cells at the margins of the foci very frequently contain two or three nuclei, and recently dead cells may show clearly the remains of as many as five or six small nuclei in a definitely enlarged cell-body. Such nuclear multiplication is greater than that commonly observed in the vicinity of necrotic patches caused by chemical poisons, and possibly represents a specific action of the virus. The adrenal foci lie in the cortex, but adjacent medullary cells contain inclusions, as do sympathetic

nerve-cells at the cortico-medullary junction. Small necrotic foci are present in the splenic pulp. A fold of peritoneum over the pancreas is the site of specific lesions, and thrombosis of a small arterial branch in the interstitial tissues is associated with specific alterations in surrounding cells. The nervous system is normal.

Intravenous Inoculation.

Rabbits inoculated with both large and minimal doses of virus display necrotic foci in various organs.

The adrenal cortex is affected in each of 6 animals. Two of these show also foci in the liver and spleen, while another died as a result of duodenal perforation. In the last the (specific) lesion responsible for rupture is only one of many in the stomach and intestines; earlier changes appear in the deeper layers of the submucosa and spread in both directions, but more especially inwards. No foci are present in the kidney, lung, heart or testis of any of the 6 rabbits.

In the nervous system of two animals killed at the onset of paralysis no lesions are present in brain, brain-stem, cervical or lumbar enlargements or intervertebral ganglia. Early lesions in the dorsal segments of the cord and in the dorsal spinal ganglia point to an ascending infection from affected viscera.

Intraperitoneal Inoculation.

In one rabbit inoculated by this route macroscopic changes in the peritoneum, so conspicuous in the monkey, are wanting. Necrotic foci are present in the adrenal, ovary, liver and spleen, but not in the kidney, lung, pancreas or mesenteric lymph-nodes. As in the monkeys inoculated by this route, in the liver and spleen the majority of the damage is immediately subcapsular; though but few inclusions are seen in the covering mesothelium, inclusions and pyknotic and karyorrhectic nuclei are numerous in the cells of the capsules.

In concluding, we may state that in the rabbit systemic infection bears considerable resemblance to that in the monkey. Ascending neuritis from peripheral infective foci is, however, very much more severe, and the central nervous system is much more susceptible, with the result that a fatal ascending myelitis ensues. In the cord the primary attack is on the ectodermal tissues, and changes in the mesoderm are mainly secondary and reactive. This probably depends on the mode of introduction of virus into the central nervous system, for after intracerebral inoculation both ectoderm and mesoderm are directly attacked by the virus, though the former is relatively more, and the latter less severely affected than in the monkey.

DISCUSSION.

Like pseudorabies and herpes viruses, the B Virus is pantropic in the sense previously indicated (Hurst, 1934), conditioning specific lesions in cellular derivatives of all three embryonic layers. In the rabbit its pronounced neurotropism is responsible for the constant and invariably fatal invasion of the nervous system by the neural route from the site of inoculation. In a given case, the number and distribution of visceral necrotic foci must obviously be related to the amount of virus entering the blood-stream, and therefore, under most circumstances, to the amount of multiplication of the virus locally in the inoculated area. Intratesticular inoculation, known in the case of other diseases to be especially favourable for virus multiplication, is followed by

early and extensive visceral infection at a time preceding the development of nervous changes. With intramuscular or intradermal inoculation only a few early foci may be present at death in the adrenal and ovary, both of which are apparently sites of election for such lesions. It has yet to be determined whether the blood-spread is readily apparent as in the case of the Aujeszky strain of pseudorabies, or demonstrated with difficulty as with the Iowa strain when recently isolated. Although, as far as the nervous system is concerned, histological evidence is indubitably in favour of infection by the neural route, it seems equally certain that, as in pseudorabies, severance of the direct nervous connection would not effectively protect the nervous axis from invasion by the virus; under such circumstances the visceral lesions would constitute the starting-point for ascending neuritis by another route.

So close is the resemblance in the rabbit between the individual lesions in this disease, herpes and pseudorabies, that only consideration of the total pathological picture permits differential diagnosis along the following lines.

After intracutaneous, subcutaneous and intramuscular inoculation, the virus of pseudorabies spreads within fifty hours or so to the spinal cord and ganglia (Hurst, 1933, 1934). In the former the posterior horns are definitely involved, while the anterior horn-cells still show no inclusions at death. So rapid is further extension to the highly susceptible medullary centres, that death occurs before appreciably inflammatory reaction has time to develop in the ganglia and cord, and, indeed, before many of the infected nerve-cells have reached the stage of necrosis. The fulminating clinical course can also account for the usual absence of visceral lesions, though virus may be present in certain organs. In keeping with this pathology, the cardinal signs of the disease are biting and scratching of the local lesion followed within a very few hours by respiratory failure.

The B Virus, after intracutaneous and intramuscular inoculation, affects the anterior horn-cells almost as quickly and quite as severely as the posterior horn and ganglia; the final picture is one of total myelitis. Ascent to the vital centres occurs far more slowly than in pseudorabies and allows time for the complete destruction of almost every nerve-cell in the first-invaded ganglia and segments of the spinal cord; in the former abundant inflammatory exudate appears. The longer course of the disease as a whole enables virus deposited in the viscera to produce necrotic lesions. On the clinical side, inconstant biting of the local lesion is followed by paralysis, with which the rabbit survives for two days or thereabouts before respiratory failure ends the scene.

Our acquaintance with the details of the herpetic lesion is more limited. In a number of cases in which intracutaneous inoculation of the Perdrau E.L.1 strain gave rise, 9 to 12 days later, to nervous symptoms, no paralysis developed; the clinical signs were those of cerebral involvement (convulsions, etc.), suggesting that the anterior horns were only slightly affected. Other workers using different strains have, however, reported paralysis (*e. g.* Goodpasture and Teague, 1923). Following intracerebral inoculation the herpes virus is responsible for more nerve-cell necrosis and cellular reaction than is the B Virus, and, of course, that of pseudorabies; at death meningeal infiltration is predominantly mononuclear, the walls of the meningeal vessels are

not necrotic, and the meningeal cells do not contain abundant inclusions. Possibly because less pronounced viscerotropism permits less marked multiplication at the site of inoculation, and at the same time the resistance of the organs is rather greater, necrotic visceral foci are not characteristic of the disease under usual conditions of study. By intravenous inoculation, however, necrosis may be brought about in the adrenal (Smith, 1931) and liver (personal observation and Smith, 1931). An interesting difference in the behaviour of the two viruses concerns the disappearance of inclusion bodies In herpes when nervous symptoms develop (as early as 6 days) after intravenous inoculation of the H.F. strain of the Rockefeller Institute, inclusions are no longer present in and around the visceral herpetic foci, while at the corresponding stage of infection with B Virus they may still be abundant. Andrewes (1930) has already remarked on the early disappearance of nuclear inclusions from the herpetic testis. As further evidence of the slighter tendency to significant infection of the blood-stream with herpes virus several observers (Goodpasture and Teague, 1923; Levaditi, 1926) have found that inoculation into a denervated area does not lead to nervous involvement and death.

In both B Virus disease and in pseudorabies the *rhesus* monkey reacts in a manner different from the rabbit. In the former the difference between the two species is relatively small, reflecting the lesser affinity of the virus for the nervous system, and its greater affinity for other tissues of the monkey; nevertheless it is the determining factor in the favourable clinical course of infection in the simian. Following intraperitoneal inoculation an adhesive peritonitis develops in the monkey but not in the rabbit. In pseudorabies, on the other hand, divergence is greater and is in the opposite direction, in that in the monkey this virus manifests only neurotropic properties (Hurst, 1933, and unpublished observations). Up to the present time *rhesus* monkeys have not been infected with herpes virus.

The theoretical implications of some of the histological findings in the rabbit are of interest. Experimental studies during recent years have led to revision of opinion regarding the significance of perivascular and meningeal infiltration in neurotropic virus diseases. Earlier workers, in the case of poliomyelitis, for example, mostly considered meningitis the primary lesion spreading by the perivascular spaces to the nervous tissues. Of necessity this theory demands a direct attack of the virus on the mesoderm; yet, on this basis, it is difficult to account for the apparent strict neurotropism of the virus, and for the lack of significant changes in viscera other than the nervous system. As long ago as 1911, however, Landsteiner, Levaditi and Pastia found that in the experimental disease the neurons might show damage in the absence of any perivascular infiltration. Hurst (1929), Fairbrother and Hurst (1930), André-Thomas and Lhermitte (1929) and Spielmeyer (1932) have confirmed the lack of dependence of parenchymatous on mesodermal lesions; under certain circumstances nerve-cell degeneration precedes, or develops *pari passu* with interstitial change. Again, as Spielmeyer points out (1930, 1932), inflammatory phenomena in the nervous system accompany many degenerative processes, and perivascular and meningeal infiltration are not, therefore, in themselves evidence of direct attack of the virus on the mesoderm. Pette, Demme and Környey (1932) and Környey (1933) maintain that mesodermal inflammation

always precedes demonstrable damage to the parenchyma; nevertheless, they express quite definitely their belief that virus multiplies only in the ectodermal tissues, which in this sense are primarily affected.

Leaving the particular, it is to-day possible to consider the question in the light of knowledge of virus diseases in general. In many such diseases of the nervous system it is not infrequent to find acute necrosis of nerve-cells in the absence of all vascular, meningeal and tissue infiltration, *provided only that the animal be killed at a suitable stage before the latter have time to appear.* Commonly in pseudorabies in the rabbit (Hurst, 1933) the course of the disease is such that death occurs before the complicating mesodermal phenomena

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DESCRIPTION OF FIGURES.

FIG. 1.—Monkey. Subcapsular necrosis in liver following intraperitoneal inoculation. Hæmatoxylin and eosin.

FIG. 2.—Monkey. Necrosis of splenic pulp (above and to right). The Malpighian bodies are almost unaffected. Hæmatoxylin and eosin.

FIG. 3.—Monkey. Necrosis in mesenteric lymph-gland. Giant-cell formation and epithelioid cells at the margin of the necrotic area. The giant-cell below and to the right is undergoing necrosis. No evidence of tuberculosis in this or other similar cases; moreover this histological picture is not characteristic of tuberculosis in the monkey. Phloxine-methylene blue.

FIG. 4.—Monkey. Lesions in a severely affected part of the cerebral cortex following intracerebral inoculation. The meshes of the pia arachnoid are filled with fibrinous exudate containing a few karyorrhectic polymorphs. The wall of the small meningeal vessel is largely necrotic and sparsely infiltrated with similar cells. The underlying cortex is the seat of vascular changes, polymorphonuclear infiltration, hæmorrhages and so on. Hæmatoxylin and eosin.

FIG. 5.—Monkey. Adventitial proliferation in a cerebral vessel closely adjacent to the area depicted in the preceding figure. Hæmatoxylin and eosin.

FIG. 6.—Rabbit. Ascending neuritis of the sciatic trunk following inoculation intramuscularly in the calf. Hæmatoxylin and eosin.

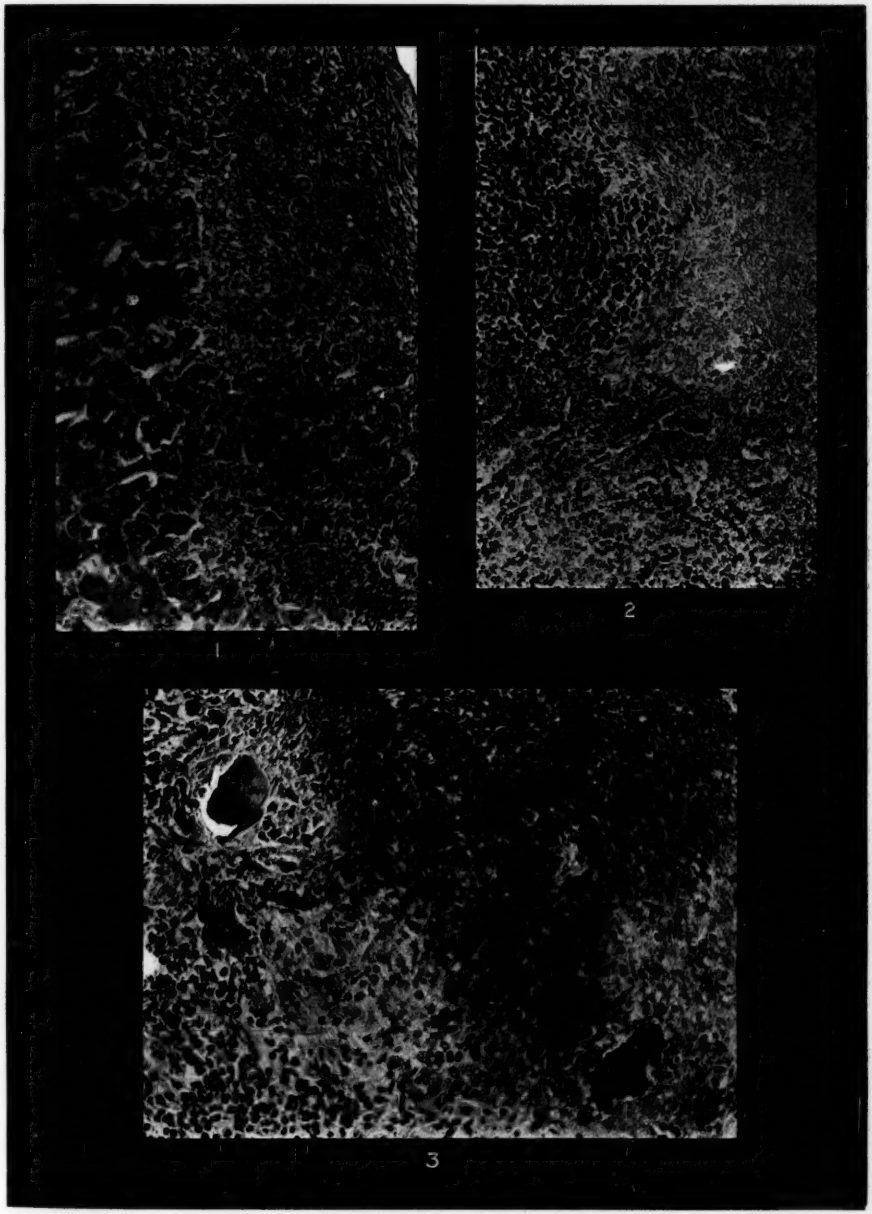
FIG. 7.—Rabbit. Relatively early involvement of a spinal ganglion related to the site of inoculation. The ganglion-cells are degenerating and contain intranuclear inclusions, or have succumbed and been replaced by nodules of phagocytic elements. Pronounced interstitial infiltration. Hæmatoxylin and eosin.

FIG. 8.—Monkey. Intranuclear inclusions in bi-nucleated cells at the advancing edge of a focus of necrosis in the liver. (*a*) Compact, strongly acidophilic type; (*b*) granular, weakly acidophilic type. Phloxine-methylene blue.

FIG. 9.—Monkey. (*a*) Inclusions in a group of proliferated epidermal cells from a skin-lesion about to become vesicular. (*b*) Inclusions in two glial nuclei from the region shown in Fig. 4. Phloxine-methylene blue.

FIG. 10.—Monkey. One of the giant-cells in Fig. 3. It contains an inclusion in each of its numerous nuclei, all of which cannot be brought into focus together. Phloxine-methylene blue.

FIG. 11.—Rabbit. Early affection of an anterior horn-cell of the spinal cord. The black intranuclear masses are basophilic remains of the nucleolus. The dark grey material represents a strongly acidophilic inclusion. Phloxine-methylene blue.



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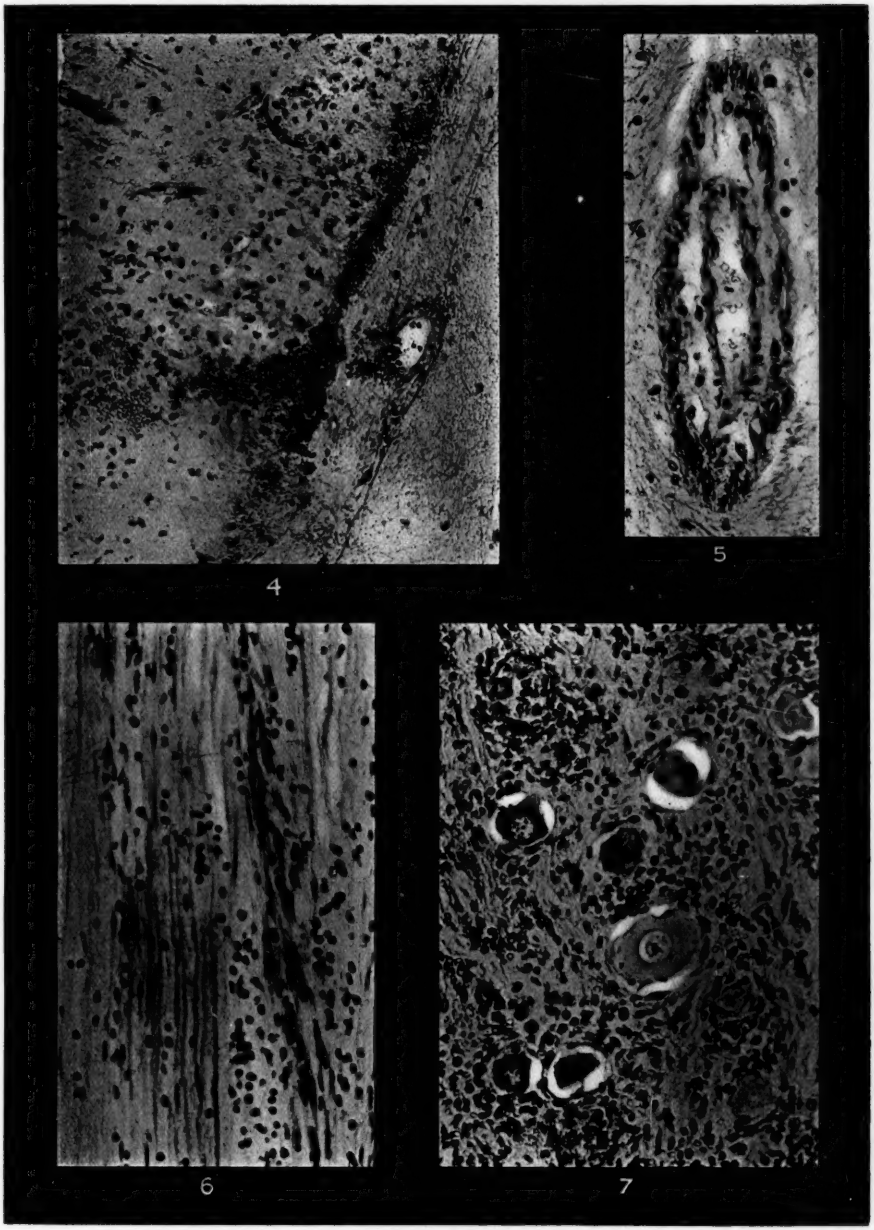

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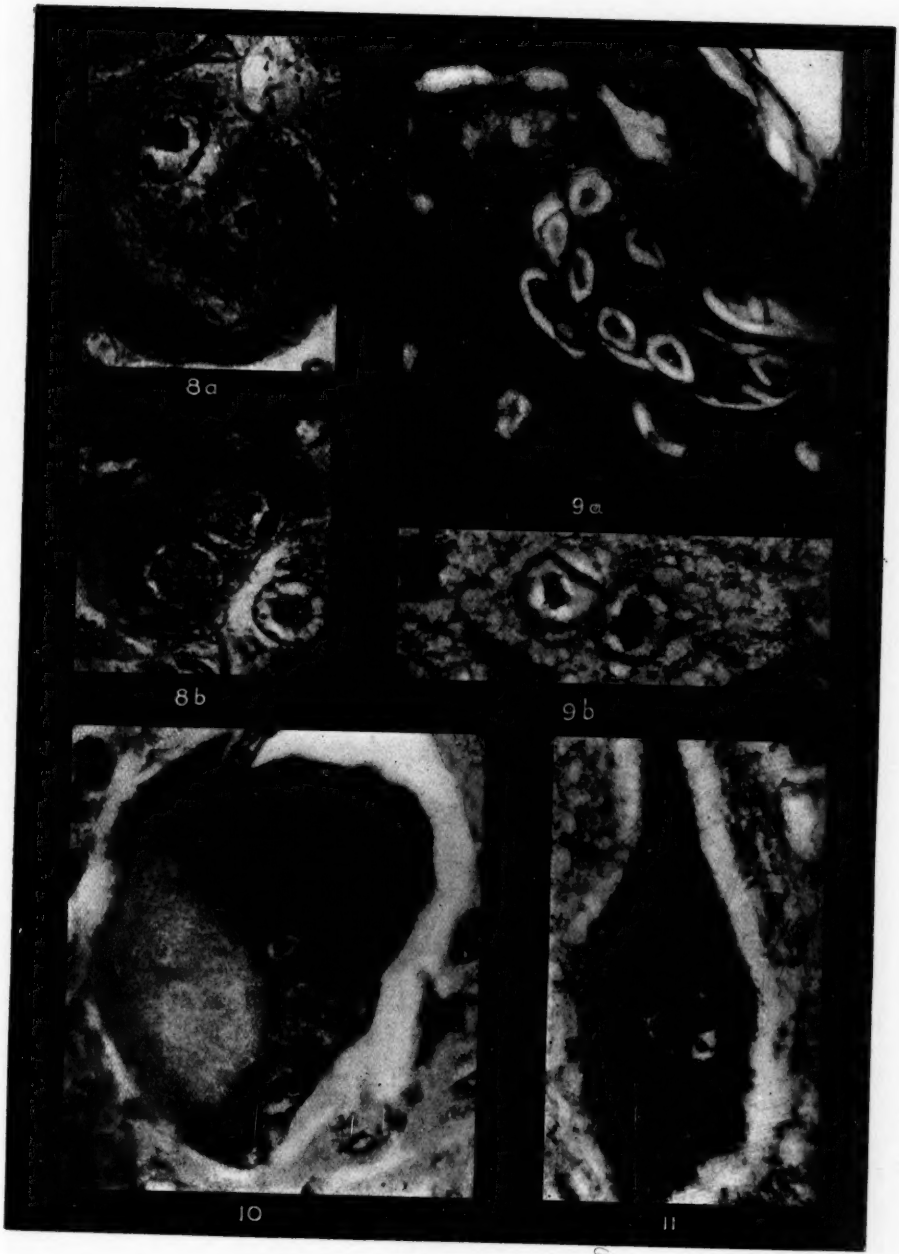

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make their *début*; the appearances are then quite unequivocally those of a primary affection of the parenchyma. In the guinea-pig the rather longer duration of the infection (greater resistance of the animal) accounts for the intrusion of these later manifestations. In the still more resistant pig interstitial reaction dominates the picture. In B Virus disease at an early stage the histological picture in the spinal ganglia may be identical with that in pseudorabies; later, as has been mentioned, the most intense reaction follows, and examination at this stage alone could not possibly lead to a decision that the nerve-cell change is primary in time as well as in the sense of Pette and his colleagues.

Now both B and pseudorabies viruses, as their behaviour in other organs testifies, are capable of a direct attack on the mesoderm. After intracerebral inoculation the possession of this property is confirmed by the specific inclusions and even by actual necrosis in vessels and meninges, appearances quite different from any accompanying infection with the more strictly neurotropic viruses. On the other hand, in the cord following peripheral inoculation of virus, the appearances in vessels and meninges can be interpreted as largely reactive in nature, even though sufficient time is allowed for the development of specific changes by any virus present. This has already been taken as possibly indicating that virus is introduced into the nervous tissues by the axis-cylinders, and that it is in response to the severe parenchymatous damage that the meningeal changes develop. The observation suggests, even if it does not prove, that in poliomyelitis and other conditions caused by the more strictly neurotropic viruses the mesodermal inflammation is in the nature of a reaction at a distance. For, on the one hand, there is in poliomyelitis a complete absence in the mesoderm of changes such as are usually associated with the direct attack of a virus on a tissue, while on the other, even in infections with viruses of proved mesodermal affinities, the parenchyma may under certain conditions be infected in such a manner as to leave the meninges, etc., uncontaminated for a time and free to develop purely reactive changes.

SUMMARY.

In the *rhesus* monkey the B Virus produces lesions characterized by the formation of acidophilic intranuclear inclusions, and later by cellular necrosis and inflammatory reaction. Lesions can probably develop in any and every tissue or organ, but under the experimental conditions here considered were found in the skin, liver, spleen, adrenals, ovary, lymph-nodes and nervous system. Intracerebral inoculation is rapidly fatal, usually with more marked affection of the membranes than of the nervous parenchyma; after peripheral inoculation there is relatively little tendency to invasion of the central nervous system.

The disease in the rabbit is similar to that in the monkey save that adhesive peritonitis does not follow intraperitoneal inoculation and peripheral inoculation leads constantly to ascending infection of the nervous axis.

The points of distinction between B Virus disease, pseudorabies and herpes infection are indicated, and the theoretical significance of the lesions in the rabbit nervous system discussed.

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A QUANTITATIVE COMPARISON OF THE EFFECTS OF THE BETA RAYS OF RADIUM ON THE AGENT OF THE ROUS SARCOMA, ON THE BACTERIOPHAGE, ON TETANUS TOXIN AND ON CERTAIN BACTERIA, ANTIBODIES AND FERMENTS.

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Received for publication February 25th, 1935.

IN previous papers (Baker and Peacock, 1926; Baker and Nanavutty, 1929) the action of ultra-violet radiation on the Rous sarcoma agent and on various organisms and ferments was studied quantitatively. The main conclusions as regards the relative susceptibilities of these bodies to the destructive effects of the rays were as follows: (1) The bacteriophage is in the same group as bacteria. (2) The Rous sarcoma agent is considerably less susceptible

* Much of the experimental work here reported was carried out at the Bland-Sutton Institute, Middlesex Hospital.

than are bacteria. (3) The various ferments examined were much more resistant than either bacteria or the Rous agent.

In these experiments the whole radiation of the mercury vapour spectrum was used. More recently, however, the experiment has been repeated by Sturm, Gates and Murphy (1932), using much more elaborate apparatus, by means of which the effects of a series of narrow wave-length bands could be studied and compared. These workers give a chart in which the effect on the Rous sarcoma agent is compared with that on *Staphylococcus aureus*, bacteriophage and vaccinia. The total energy, in ergs per sq. mm. of exposed surface, necessary to produce a 50 p.c. reduction in numbers or potency is charted at various wave-lengths between 2380 and 3130 Å. These results confirm our general findings, and show that the staphylococcus, bacteriophage and vaccinia virus fall into a group at about the same energy level, while the curve for the Rous sarcoma agent indicates that on the average rather more than ten times the energy is required for its destruction. In addition the point of maximum efficiency in the spectrum appears somewhat different in the case of the Rous agent, but it is not clear what significance can be attached to this observation.

The resistance of the sarcoma agent to ultra-violet rays does not appear to be connected with the small size of its particle, since, according to estimates by Elford's filtration method (Andrewes, 1934), this exceeds that of the largest bacteriophage.

More recently Duggar and Hollaender (1934) have shown that one of the plant viruses (tobacco mosaic virus) is highly resistant to destruction by ultra-violet rays—apparently considerably more resistant than the Rous sarcoma agent.

As a further study along the same lines the present work on the action of the beta rays of radium was undertaken.

PREVIOUS WORK.

A considerable amount of work has been done on the action of radium on bacteria, viruses, protozoa and ferments, and it has been shown that, as might be expected, these bodies are all susceptible to the destructive effects of its rays if applied in sufficient doses.

It will, perhaps, be advisable for the benefit of those who have not had occasion to consider the destructive action of radium to summarize briefly the relevant facts concerning the three types of rays emitted.

Alpha rays consist of positively charged helium atoms emitted at high velocity. They are highly destructive, but of such low penetrating power that they are stopped by a thin layer of glass, aluminium or even paper. They are, therefore, not emitted by the usual types of applicator, but can be applied to a suspension of bacteria or other fluid by adding a known quantity of radium emanation to the liquid and sealing up in a glass tube.

Beta rays, which consist of electrons emitted at various velocities, some approaching the speed of light, have a range of penetration which allows a high proportion of them to pass through thin glass or aluminium (0·1 mm.). They can be screened off by 5 mm. of aluminium or 1 mm. of lead. They provide an agent which has a marked destructive power (though considerably less than that of the alpha particles), and can be easily applied to bacterial suspensions or bodies in solution.

Gamma rays, consisting of electro-magnetic disturbances of very short wave-length, similar to "hard" x-rays, have a great penetrating power. Only a very small fraction of these rays

would be absorbed in passing through a small depth, (*e. g.* 1 cm.) of a bacterial suspension or similar fluid of approximately the specific gravity of water.

Since it is known from physical considerations that only those rays which are absorbed can exert destructive effects, it could be predicted that gamma rays would only have relatively slight destructive action on bacteria or solutions of ferments, etc. Such has been found to be the case. Chambers and Russ (1912) were unable to obtain any evidence of destructive action on bacteria after exposure to the gamma rays from 7 mg. of radium for a week; Bruynoghe and Mund (1925*a*) also found gamma rays bactericidally inactive. Since marked destructive effects are produced on bacteria in a few hours if the beta rays are not screened off, it is clear that the action of the accompanying gamma rays may be ignored in experiments where beta-ray effects are being studied.

Action on bacteria and viruses.—Green (1904) obtained destructive effects (beta ray effects) on *B. coli*, *V. cholerœ*, *Staph. aureus*, *B. anthracis* (sporing), *B. mesentericus*, and vaccinia. The amount of destruction was estimated in the case of bacteria by colony counts after varying exposures. By plotting Green's figures one can express the relative resistance of each of these organisms to beta rays (in the form of length of exposure necessary to reduce to 1/1000th of original numbers) as follows: *B. coli* and *V. cholerœ*, 1; *Staphylococcus*, 2; *B. mesentericus*, 8; *B. anthracis* (sporing), 11. No estimation of titre is recorded in the case of vaccinia, so that it is difficult to make comparisons with the bacterial group. If, however, one assumes that the titre lay somewhere between 10^5 and 10^2 effective doses per unit volume, with a more or less logarithmic rate of destruction, the resistance as compared with *B. coli* would be between 1 and 3, *i. e.* within the bacterial range.

Apart from this work of Green's there appears to be very little that gives information as to the relative susceptibility of different types of bacteria or other living agents. The French workers have carried out numerous investigations, which are as a rule, however, lacking in satisfactory quantitative data. It has been demonstrated that in the case of motile bacteria, trypanosomes and various protozoa motility is retained, while the power of reproduction is lost (Bruynoghe and Mund, 1925*a*; Bruynoghe and Dubois, 1925*a*, *b*, *c*.)

Bruynoghe and Le Fevre de Arric (1925) demonstrated the destruction of the viruses of encephalitis, rabies and herpes zoster by radon, but the quantitative data as to rate of destruction are not available. Lacassagne and Pauline (1925) estimated the relative susceptibility of 24 different types of bacteria to beta rays, but the procedure used—measurements of the diameters of sterilized zones on agar plates—is not satisfactory as a quantitative method. Muttermilch and Ferroux (1925) studied the effects of beta rays on neuro-vaccine, and by the same technique on tetanus toxin. The length of exposure required to inactivate was much greater in the case of the neuro-vaccine than with the toxin, but the titre of the former was 10^8 M.L.D., while that of the latter was 10^4 M.L.D., and as no attempt was made to estimate the percentage reduction in either case it is difficult to make comparisons. However, if one allows for the initial difference in titre and assumes a more or less logarithmic rate of destruction, it does appear that the toxin was more susceptible to irradiation than the virus.

Action on ferments.—The fact that most of the earlier workers failed to demonstrate destructive effects of beta rays on ferments suggests that the enzymes would prove more resistant than bacteria.

The only papers in which I have been able to find any adequate quantitative data are those of Hussey, Thompson and Northrup. Hussey and Thompson carried out a most careful series of experiments, in which they made quantitative estimations of the destructive effects of beta rays on various digestive ferments. In their paper on trypsin (Hussey and Thompson, 1922) they showed that the amount of ferment destroyed is a function of the millicurie hours of radiation, the logarithm of the concentration being directly proportional to millicurie hours' exposure. Similar work on pepsin (Hussey and Thompson, 1923) and on invertase (Hussey and Thompson, 1925) has established the same facts in these cases, and has shown that both these enzymes are much more resistant to destruction than is trypsin. From these workers' figures it can be shown that, in order to produce the same percentage reduction, pepsin requires about 35 times, and invertase over 60 times the exposure necessary in the case of trypsin.

In the experiments reported below I have been able to produce an easily measurable destruction of trypsin, but with the small amount of radium available it would have required impracticably long exposures to obtain measurable effects on pepsin or invertase. The figures for these can, however, be calculated from the above ratios.

TECHNIQUE.

The same standard source of rays has been used for all the experiments here reported, namely a circular capsule* containing 14·9 mg. of radium element spread over a circular area 1·7 cm. in diameter, and covered by a disc of aluminium 0·1 mm. in thickness. The capsule was arranged so that its surface formed the floor of a small chamber sealed with a paraffined glass plate to prevent evaporation. The material under treatment was always contained in 0·15 c.c. of fluid lying in the form of a large drop on the surface of the capsule, which was greased with a trace of vaseline.

Since the Rous sarcoma agent deteriorates rapidly at room temperature, exposures were carried out in the refrigerator at 0° C. except in the case of complement, which was kept frozen solid. Untreated control material was kept at the same temperature in the form of a standard drop in a similar chamber on a dummy capsule with a greased aluminium surface.

The effects of beta rays on the following have been examined: *B. coli*, *Staphylococcus aureus*, *B. anthracis* (spores) bacteriophage (the same strain as used in ultra-violet experiments previously reported), Rous sarcoma No. 1 (Berkefeld filtrate), hæmolytic amboceptor, guinea-pig complement, trypsin, lysozyme and tetanus toxin. With the exception of the tetanus toxin the methods of estimating quantitatively these various organisms have already been described (Baker and Nanavutty, 1929).

Tetanus toxin was added to the list because the work of Muttermilch and Ferroux quoted above pointed to the fact that it was rather easily destroyed. A solution of the toxin in 50 p.c. glycerine was diluted 1 in 20 before setting

* Kindly lent to me by Prof. S. Russ.

up samples for irradiation and controls as described. After varying exposures samples of toxin were inoculated into mice in falling dilutions and the effects as regards M.L.D., and production of leg spasm with recovery were matched against dilutions of the control sample. The M.L.D. of the control sample (1/20 of original solution) was approximately 0·005 c.c. It was found that it

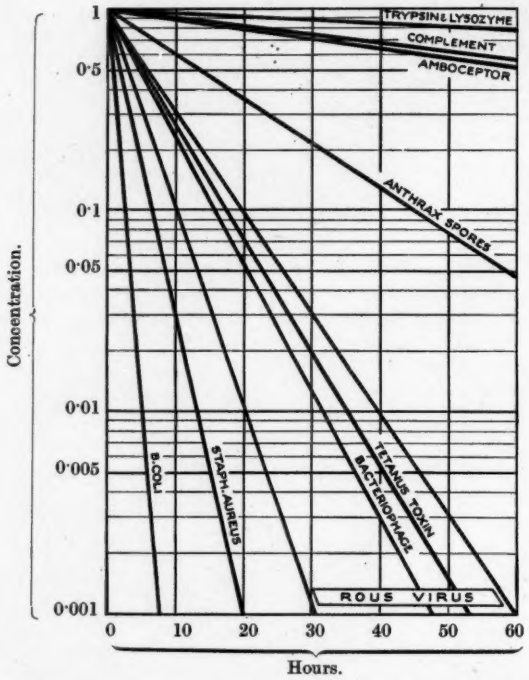

FIG. 1.—Graph showing the effects of beta rays on the titre of the various bodies examined. The ordinates (logarithmic ruling) show the fractions remaining after various exposure times. A wide margin is allowed in the case of the chicken sarcoma agent in view of the difficulty of precise estimation of titre.

took about 18 hours' irradiation to reduce the toxin to 1/10th of the titre of the control sample.

In the case of the Rous filtrate irradiated and control samples were inoculated into opposite sides of the same bird in each case. Several different batches of filtrate were treated with various lengths of exposure, but the precise diminution of titre could not be assessed with the same accuracy as with the other agents treated. The results are therefore expressed as lying between certain values (see figure).

RESULTS.

The results of these experiments are shown in the table in the form of the number of hours of irradiation required to reduce the numbers or concentration to the fraction indicated at the head of each column. It was obviously necessary to give numerous exposures in each case, but the levels of concentration charted were those which appeared to give the most clear-cut results. In the case of bacteria, for instance, it was easy to read the results at almost any level, while in the case of the bacteriophage a well-defined reduction to 1 : 1000 of the original concentration appeared most reliable. In the cases of antibodies and ferments only slight reductions were obtained, even with lengthy exposures.

TABLE I.—*The Number of Hours of Irradiation necessary to Reduce Various Bodies to the Concentrations indicated above; the Initial Concentration being taken as Unity.*

In the last column is shown the ratio of the exposure times necessary to produce an equal percentage reduction in each case, taking the figure for *B. coli* as unity. The figures for pepsin and invertase are calculated from Hussey and Thompson's results, that for vaccinia from Green's figures.

	Concentration after irradiation.						Exposure time ratios.
	0·85.	0·7	0·5.	0·1.	0·01.	0·001.	
B. coli	..	..	..	2¼	5	10	1
Staphylococcus aureus	..	..	..	7	..	20	2·5
Vaccinia	..	..	..	..	..	..	1–3
Bacteriophage	..	..	..	..	..	50	5
Rous sarcoma filtrate	..	..	..	12–24	..	..	5–10
Tetanus toxin	..	..	..	20	..	..	9
B. anthracis spores	..	..	..	45	..	..	20
Hæmolytic amboceptor	..	..	66	..	..	..	100
Guinea-pig complement	..	45	..	..	..	..	130
Trypsin	42	93	..	..	..	..	250
Lysozyme	..	95	..	..	..	..	250
Pepsin	..	..	..	..	..	..	9000
Invertase	..	..	..	..	..	..	15,000

The last column of this table gives the ratios of the resistances of the various bodies—that is, of the exposure times producing a corresponding percentage reduction in each case; that for *B. coli* was taken as unity. The figures for pepsin and invertase are based on Hussey and Thompson's results quoted above. In the case of anti-bodies and ferments, where only slight reductions are obtained, the assumption is made that the rate of destruction is approximately logarithmic. This has been shown to be true in the case of the digestive ferments (Hussey and Thompson, *loc. cit.*), and my present results with *B. coli* and staphylococcus indicate that it is true for bacteria; in the case of the other substances here studied it is a reasonable but unproved assumption.

The figure sets forth the results in graphic form plotted so that the ordinates are proportional to the logarithms of the concentrations. The range allowed

for the Rous sarcoma agent is shown by the space between the lines which end at the 30- and 60-hour marks.

DISCUSSION AND CONCLUSIONS.

It is seen that as regards susceptibility to destruction by beta rays the Rous sarcoma agent falls within the bacterial group. On the other hand, tetanus toxin also falls within this range, so that it is obviously not possible to deduce that the Rous agent is a living organism on the strength of these findings.

On the other hand, the ferments so far examined are much more resistant to destruction than the most resistant organism tested (anthrax spores). Recent work by Northrup (1934) on the destruction of crystalline pepsin by beta rays has shown that the loss of activity parallels the disappearance of protein nitrogen from the solution. This, together with its high resistance to destruction, suggests that a relatively gross alteration of the molecule is necessary in order to destroy the activity.

Trypsin is less resistant than the other ferments examined, but it still requires 25–50 times the dose necessary in the case of the Rous agent and at least 10 times the dose for anthrax spores. In these experiments the trypsin was diluted and at pH 7·5. (Strong trypsin in the acid state showed no evidence of any destruction with ten times the maximum period of irradiation indicated in the table. Hussey and Thompson (1922) also record this observation.)

Although these results do not afford evidence as to the living nature of the Rous sarcoma agent, they do point to the fact that it differs widely in its make-up from the ferments so far examined. It appears, therefore, very improbable that this agent is a ferment in the accepted sense of the term.

SUMMARY.

1. A quantitative study has been made of the destructive effects of the beta rays of radium on the following bodies: *B. coli*, *Staph. aureus*, bacteriophage, filtrate of Rous sarcoma No. 1, tetanus toxin, *B. anthracis* spores, hæmolytic amboceptor, guinea-pig complement, trypsin and lysozyme.

2. The relative susceptibility of these various bodies to destruction by beta rays has been estimated, and in addition figures for vaccinia, pepsin and invertase are deduced from other workers' results.

3. The agent of the Rous sarcoma fell into the bacterial group, but tetanus toxin was equally susceptible.

4. The antibodies were considerably more resistant to destruction and the ferments still more resistant.

5. The evidence of these results as to the living nature of the Rous sarcoma agent was equivocal, but it did point to the fact that this agent was not of the nature of a ferment.

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A MOTILE STREPTOCOCCUS.

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Received for publication February 22nd, 1935.

MOTILITY has been chiefly associated in the minds of bacteriologists with bacilli, though a few cocci have been reported as possessing this property. *Micrococcus sensibilis, Sarcina citrea, Sarcina ureae* and *Rhodococcus agilis* are described by Bergey (1923) as being motile. Streptococci of all types have, however, generally been considered non-motile.

This paper describes a type of enterococcus which, in certain circumstances, is actively motile and has been shown to possess flagella. Four strains have been isolated from the following sources:

(1) A variant of enterococcus isolated from human urine. The original strain possessed the characteristics of a typical enterococcus as described by Dible (1921).

(2) A variant of an enterococcus isolated from human fæces, also of normal type when isolated.

(3) A variant of an enterococcus isolated from the thrombosed uterine veins of a woman dead of puerperal fever. Though differing in some particulars from the normal type, the original strain was non-motile and produced no visible hæmolytic change on blood-agar.

All these variants arose spontaneously in cultures kept for some months on simple media and subcultured about once monthly. Occasionally reversions to the original types have occurred.

(4) A variant isolated from an old blood broth culture of another type of streptococcus.

The following description applies to all four strains, which appear almost identical.

OBSERVATIONS.

Morphology.—The organism is a Gram-positive coccus, lanceolate in shape and grows in pairs and short chains. No capsules have been demonstrated. Flagella are present.

Type of growth on various media.—It grows well on simple media, but rather less profusely than the usual type of enterococcus. Growth in broth is diffuse without pellicle or sediment; growth on agar is transparent and unpigmented. MacConkey's media, solid and liquid, support growth well; in pure bile slow growth occurs. On blood-agar the colonies are surrounded by the green coloration associated with *Streptococcus viridans*; this is later followed by clearing of the medium around the colony. On all media colonies are typically streptococcal in appearance.

Viability.—Cultures will survive for many weeks at room temperature.

Heat resistance.—The organisms withstand heating to 60° C. for 20 minutes, but not 30 minutes.

Carbohydrate fermentation.—Acid without gas is produced in 24 hours from mannitol. Æsculin is also fermented.

Motility.—A culture in serum broth shows active motility during the logarithmic phase of growth. The features of the movement are very like those of *Bact. typhosum*. Diplococci dart rapidly across the field with the familiar side-to-side movement. Chains of medium length generally move in a slower and more sinuous manner, resembling the filaments of bacillary cultures. Long chains of twenty or more cocci have been observed moving thus, but these are not produced when the conditions for optimum growth are present.

The following media were found suitable for the demonstration of motility. (1) Veal broth and bullock's heart broth (pH 7·6) without serum. Motility, though present, is less marked than in serum media. Some batches of broth are unsuitable. (2) Serum broth; provided serum is added, veal, heart and lemco broths have been found suitable. Rabbit, human, horse and guinea-pig sera appear equally effective; anything from a few drops to 10 p.c. serum may be added to 4 or 5 c.c. of broth; to ensure good results it is, however, essential that the serum, whatever the type, should be previously heated for 30 minutes or more at 56° C. Unheated serum appears to inhibit motility, and in the case of horse serum the organisms are clumped. The same factor probably prevents active motility in blood broth cultures.

Cultures on solid media, when emulsified in water, saline or serum show little motility.

The period of maximum motility is definitely during the logarithmic phase of growth, usually after 6–8 hours' incubation at 37° C. After 18 (sometimes after 24) hours' incubation motility, though still present, is less marked, and after longer periods is absent.

Flagella.—After many unsuccessful attempts to stain the flagella in liquid cultures, it was decided to apply a flagellar stain to a film of organisms grown on blood-agar and emulsified in distilled water, in the hope that flagella had

been produced on this media though motility was hardly demonstrable. This proved to be the case.

The following method, having been found successful in staining the flagella of *Bact. typhosum* and *Chromobacterium prodigiosum*, was used to demonstrate those of the enterococcus. Three reagents are required, of which (1) and (2) are those used by Bailey (1929) for his capsule and flagellar stain: (1) A mordant consisting of 3 parts of 5 p.c. tannic acid in water. (2) A mixture, freshly prepared, consisting of 14 drops of mordant, 2 drops of carbol fuchsin, 2 drops of strong hydrochloric acid, and 2 drops of formalin. (3) The stain. This is an ammoniacal solution of silver carbonate prepared according to the following directions: To 10 c.c. of 10 p.c. $AgNO_3$ add 40 c.c. of 5 p.c. Na_2CO_3. Allow the precipitate to settle and decant the supernatant fluid. Wash the precipitate with distilled water and re-decant. Dissolve the precipitate by adding, drop by drop, just enough strong NH_4OH to put it almost into solution, leaving 5 or 6 grey granules undissolved. Make up to 100 c.c. with distilled water.

The film, to which no heat has been applied, is treated with (1) for 2 minutes, then with (2) for 9 minutes. After that it is well washed and (3) is applied for 3–4 minutes.

It was more difficult to obtain a satisfactory picture of enterococcal flagella than of those of the various bacilli, partly because they are so much shorter and scantier, still more because of the strong tendency shown by the cocci to adhere together in tight clumps. The majority of isolated cocci or diplococci appeared to possess one flagellum. This varied in length, but was generally two or three times as long as the organism, and was attached to a point between the centre and one of the extremities. Occasionally two flagella appeared to be attached to one coccus, both arising from the same end. No diplococcus was seen with flagella at both ends. A few flagella gave an appearance of branching. Films of pneumococci and non-motile enterococci, stained in a similar manner as controls, showed no sign of flagella.

Serological reactions.—An antiserum to one of the strains agglutinated all four to the same titre, and fixed an equal amount of complement with all.

No evidence has, so far, been obtained of any flagellar antibody in this antiserum. The type of agglutination is not floccular, though the granules are of medium size and can be seen after an hour at 50° C. This, however, is the case with non-motile enterococci in strong homologous antisera. Boiling broth cultures of these motile cocci for an hour rendered them rather more agglutinable than before. Alcoholized suspensions agglutinated to a lower titre and rather more slowly than the original cultures, but it is possible that antigens other than flagellar antigen may be altered by this procedure.

SUMMARY.

A description is given of a type of enterococcus showing active motility, and of the appearance of its flagella when stained.

I am indebted to Dr. B. D. Pullinger for suggesting the use of ammoniacal silver carbonate as a flagellar stain.

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ADDENDUM.

Since this paper was sent in for publication, my attention has been drawn to the following paper, dealing with the same subject: Koblmüller, L. O., "Untersuchungen über Streptokokken. I: Über bewegliche Streptokken", *Zbl. Bakt. O.*, 1935, **133**, 310. Koblmüller describes twelve strains of motile enterococci, which appear to be, in all essentials, similar to those reported here.

THE MECHANISM OF IMMUNITY TO FILTERABLE VIRUSES. III. *RÔLE* OF LEUCOCYTES IN IMMUNITY TO VACCINIA.

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Received for publication February 25th, 1935.

IT was pointed out in the preceding communications (Sabin, 1935*a*, 1935*b*) that the mechanism of immunity to certain viruses is intimately bound up with the susceptible cell, and that the protective substance in antiviral serum can exert its effect in the absence of leucocytes in tissue-containing cultures, without destruction of the virus. It was, nevertheless, necessary to determine whether or not leucocytes were capable of destroying the virus, and thus of playing a part in its elimination. Also in view of the claims that there is an antibody which combines with the virus, leading to specific agglutination, flocculation and complement-fixation, it was essential to determine whether or not antiviral serum possessed an opsonic action on the virus. This question of the *rôle* of the leucocytes has been studied by a few investigators only thus far. Smith (1929) showed that when vaccinia virus is injected intravenously in a normal rabbit or when it invades the blood-stream from the skin, it rapidly becomes associated with the white cells, which then become infectious. He also found that when vaccinia virus is injected intravenously into an immune

rabbit, the blood-cells, obtained several hours later and washed free from plasma, could be shown to be infective. The latter observation, which was confirmed by Long and Olitsky (1930), in itself suggests that immunity could not depend upon phagocytosis, even if it occurred, since it has been amply demonstrated that vaccinia can infect susceptible cells within a few minutes (Andrewes, 1929). Douglas and Smith (1930) claimed that normal rabbit blood is viricidal for vaccinia, this property being dependent upon the leucocytes and a thermolabile constituent in the serum, and also that the blood, spleen and testis of immune rabbits showed far greater viricidal action, which they attributed in part to alterations in the cells themselves and partly to an opsonic action of the serum. It should be pointed out, however, that in view of the fact that normal tissue can fix protective substance (Sabin, 1935*b*), an examination of their protocols suggests that the steps taken to free the blood and tissue-cells from protective substance (immune serum) before testing for virus were probably inadequate. Fairbrother (1933) claimed to have shown a viricidal action of normal, and an even greater action of immune leucocytes, on vaccinia in the presence of immune serum, by means of intracerebral tests in rabbits. The data are not conclusive, however, because the total number of animals was small, the results irregular, the additional protective substance contained in the immune cells was not determined, and temperatures were not taken, the only criteria of infection being clinical signs and a fatal termination. It is not unusual for some rabbits, injected intracerebrally with vaccinia, to react only with fever and subsequent immunity to quantities of virus which cause death in others. Jamuni and Holden (1934) made similar claims in the case of herpes on the basis that serum-virus mixtures which were in themselves infectious when injected intracerebrally in rabbits, sometimes were not so when incubated with leucocytes. Their results are also inconclusive because the total number of tests was very small, and an examination of the protocols reveals that some of the rabbits receiving only virus and serum also survived. A critical survey of the reported work thus fails to provide conclusive evidence that immune serum can exert an opsonic action on the viruses studied. If, however, an opsonic action could be definitely demonstrated, there would be undoubted evidence for the existence of an antibody against the virus itself, even though that antibody might not be the agent primarily concerned in the actual protection of tissues against infection.

METHODS.

Vaccinia virus was selected for this study, first, because most of the existing work on this problem has been done with it, and secondly, because it lends itself readily to quantitative estimation without requiring large numbers of animals. The virus was used in the form of Berkefeld "V" filtrates to eliminate, as far as possible, the chances of non-specific phagocytosis of tissue particles containing virus. The experiments were always performed with known quantities of virus, and titrations of different fractions were carried out, whenever possible, on the same rabbit, in order that the results might be susceptible of at least some rough quantitative check. Furthermore, since even the same

virus suspension when titrated in duplicate on a single rabbit may give results which vary within a ten-fold dilution, it appeared unwise to work with minute amounts of virus such as 10 or even 100 M.I.D. The virus was a rabbit-testicular strain, and the antivaccinial serum was obtained from convalescent rabbits. The protective capacity of the immune serum was determined on several rabbits prior to its use in an experiment, in order to have an approximate idea of the dilution in which it might still be effective against various amounts of virus. The leucocytes were obtained either from heparinized or defibrinated blood by differential centrifugation, or from the pleural cavities of a rabbit 24 hours after injection of an aleuronat-starch-broth mixture. The leucocytes, which were always admixed with many red blood-cells, were examined and counted in a hæmocytometer. Whole blood, employed by Douglas and Smith (1930) in their studies, was not used because of the difficulty of washing the cells adequately. The total number of leucocytes, the important cells as Smith (1929) has shown, could thus be greater, and when centrifuged would, in themselves, constitute a volume of only 0·01 or 0·02 c.c., so that any loosely adherent immune serum could be readily diluted to ineffectiveness by a single washing. The virus-leucocyte mixtures were incubated at 37° C. on a mechanical shaker, at a speed which was just sufficient to prevent the cells from settling out. It was observed that when clotting occurred in a mixture it could be a source of great error ; the clot would enmesh most of the virus, and, even when it was broken up by shaking, the liquid portion would contain no virus when the original concentration was not great. If the clot was ground up with sand, however, one could show that it contained the virus. Andrewes (1928) made use of the formation of a clot to show that active virus was present in a " neutral " serum-virus mixture.

FATE OF VACCINIA VIRUS IN A MIXTURE OF FRESH NORMAL SERUM AND NORMAL LEUCOCYTES.

Although this experiment was prompted primarily by the experience of Douglas and Smith (1930), which suggested that a mixture of fresh normal serum and normal leucocytes had a viricidal effect on vaccinia, it also aimed at answering the following questions : Can fixation of virus be demonstrated with leucocytes as with minced testis ? If such fixation occurs, is it followed by destruction of the virus ? What would be the influence of varying the amount of virus in the mixtures ? Would leucocytes, which have fixed virus, be as infectious in the intact state as when broken up ?

Thirty c.c. of blood was obtained from two rabbits by cardiac puncture. It was defibrinated by shaking with glass beads, and then centrifuged lightly to throw down most of the red cells. The supernatant opalescent serum was then centrifuged at about 2000 to 3000 r.p.m. for 15 minutes. The sedimented cells, red and white, which were thus obtained were suspended in 4 c.c. saline. The virus was a Berkefeld "V" filtrate of a 5 p.c. testicular suspension, and had a titre of 1 : 100,000 when 0·2 c.c. was injected intracutaneously in a rabbit ; the virus was titrated with the same result on the rabbits used in this experiment. The mixtures were set up as follows :

Ampoule No.	Leucocyte suspension.	Fresh normal serum.	Virus filtrate.	Incubation at 37° C.
I	1 c.c.	0·44 c.c.	0·16 c.c. undiluted	2 hours.
II	,,	,,	Ditto	20 ,,
III	,,	,,	0·16 c.c. of 1 : 10 dilution	2 ,,
IV	,,	,,	Ditto	20 ,,
V	None ; saline, 1 c.c.	,,	0·16 c.c. undiluted	20 ,,

The ampoules were sealed in the flame and put on a shaking machine at 37° C. for the indicated periods. Ampoule V was set up to control the effect of shaking the virus at 37° C. for 20 hours. After incubation, the cells were

TABLE I.—*Fate of Vaccinia Virus in a Mixture of Fresh Normal Serum and Normal Leucocytes.*

Amount of virus in serum-leucocyte-mixture.	Fraction tested.	Dilution.	Result. After 2 hours at 37° C.	Result. After 20 hours at 37° C.
About 80,000 M.I.D.	Supernatant liquid	Undiluted	++++	+++++
		10^{-1}	+++	++++
		10^{-2}	++	+++±
		10^{-3}	+±	++
		10^{-4}	−	−
	Cells unground	10^{-2}	++++	+++++
		10^{-3}	+++	++++
		10^{-4}	++±	++++
		10^{-5}	+	+++
	Cells ground with sand	10^{-2}	++	+++
		10^{-3}	+	+++
		10^{-4}	±	++
		10^{-5}	−	−
About 8000 M.I.D.	Supernatant liquid	Undiluted	+++	+++±
		10^{-1}	++	+++
		10^{-2}	+	++
		10^{-3}	−	−
	Cells unground	10^{-2}	+++±	+++++
		10^{-3}	+++	++++
		10^{-4}	++	+++
		10^{-5}	−	+
	Cells ground with sand	10^{-2}	+++	+++
		10^{-3}	++	++
		10^{-4}	±	−
		10^{-5}	−	−

centrifuged at about 3000 r.p.m. for 10 minutes, and the supernatant liquid was drawn off. The sedimented cells measured approximately 0·02 c.c. by volume and enough saline was added to dilute them 1 : 100 ; half was used as such for further dilutions. The other half was ground with a small amount of sand, and after allowing the coarse particles and sand to settle out, further dilutions were made from the supernatant liquid. The different fractions were injected into the rabbits as soon as they were obtained, one rabbit being used for the 2-hour set, and another, the following day, for the 20-hour set. The results are shown in Table I.

It may be seen from the table that in each case the leucocytes had approximately 100 times more virus per unit volume than the supernatant liquids, and even though they were not washed, this may be taken as evidence of fixation of the virus by the cells. It is interesting to note that although the number of leucocytes was nearly the same in all mixtures, the amount of virus per unit volume of the cells was not the same, but varied almost in direct proportion to the amount of virus in the mixture. It should also be noted that the amount of virus present in the different fractions after 20 hours at 37° C. is the same as after 2 hours at 37° C. ; the larger lesions recorded for the 20-hour titration are most likely due to rabbit variation. The titration of the " temperature and shaking " control, which is not recorded in the table, revealed that there was no spontaneous deterioration of the virus during that time. It is quite apparent that fresh normal serum and normal leucocytes had exerted no viricidal effect, and that leucocytes which fix virus become highly infectious. When one compares the results obtained by injecting intact leucocytes and those which were ground with sand, it is evident that considerably more virus was recovered from the intact ones. This was contrary to expectation, and the results are probably due to the fact that virus remained adsorbed to the cell *débris* which settled out with the sand.

FATE OF VACCINIA VIRUS IN A MIXTURE OF NORMAL LEUCOCYTES AND IMMUNE SERUM, AND THE FIXATION OF ANTIVIRAL SUBSTANCE BY LEUCOCYTES.

In the preceding experiment it appeared that leucocytes, like minced testis, were capable of fixing virus, but that following such fixation the virus was not destroyed. The purpose of the present experiment was to repeat the former with certain modifications, and to determine whether fixation of virus would occur in the presence of immune serum, and whether the virus would be destroyed. In view of the experience in the preceding investigation (Sabin, 1935*b*), which showed that minced testis could fix antiviral substance, it was realized that the experiment must be controlled to show whether and to what extent leucocytes would do the same.

The leucocytes were obtained from the pleural cavity of a rabbit 24 hours after inoculation with an aleuronat-starch-broth mixture. The blood-tinged exudate was mixed with an equal volume of 1 : 1000 heparin in saline. This suspension contained about 15,000 leucocytes per c.mm., most of which were polymorphonuclear. The virus filtrate had a titre of 1 : 10,000, and when

mixed with an equal amount of the undiluted immune rabbit serum used in this experiment, gave no lesion when injected intracutaneously. The experiment was set up as follows :

Ampoule.	Leucocytes in fresh heparinized plasma.	Virus.	Serum.
I	1·2 c.c.	0·15 c.c.	0·15 c.c. normal rabbit.
II	,,	,,	,, immune ,,
III	,,	None; 0·15 c.c. Tyrode only	,, ,, ,,

After incubation at 37° C. for 3½ hours, samples were removed from ampoules I and II to test the effectiveness of the immune serum on the same rabbit. All the mixtures were then centrifuged at about 3000 r.p.m. for 10 minutes, and the supernatant liquids drawn off. The sedimented cells measured approximately 0·01 c.c. by volume ; 5 c.c. of saline were added to each tube, and the sediment redispersed with a pipette, so that the dilution of any loosely adherent matter would be 1 : 500. All tubes were again centrifuged, and the supernatant liquid discarded. The cells of tubes I and II were taken up in 1 c.c. saline each, and those of tube III in distilled water ; this gave a dilution of 1 : 100 by volume for the cell suspensions, and a final dilution of any freely dispersible virus or serum of 1 : 50,000. In view of the experience in the preceding experiment, the cells from tubes I and II were not ground. The cells which were taken up in distilled water were frozen and thawed 10 times, when an examination revealed no intact cells. This suspension was then mixed with equal amounts of varying dilutions of virus filtrate and injected intracutaneously on the same rabbit to test for the presence of antiviral substance. The leucocytes " treated " with the immune serum were broken up by freezing and thawing to avoid the possible criticism that they might have acted on the added virus in the interval preceding inoculation or *in vivo*. The results of this experiment are shown in Table II.

The results obtained with the fractions of the normal serum mixture confirm those of the first experiment, and since the cells in this case were washed, the fixation of virus is even more definitely established. It is also quite clear that the virus is not destroyed. The mixture of immune serum, virus and leucocytes contained enough protective substance to prevent a lesion when the total mixture was injected, but the cells, centrifuged from it and washed, were infective in a dilution of 1 : 100. It is evident, therefore, that (*a*) the virus was not destroyed by the leucocytes in the presence of an excess of immune serum, and (*b*) the leucocytes fixed virus in the presence of immune serum. But whereas the cells from the " normal " mixture had a titre of 1 : 10,000, those from the " immune " mixture had a titre of only 1 : 100. An examination of the protocols of Douglas and Smith (1930) reveals qualitatively similar data, *i. e.* infectivity of blood-cells centrifuged from a non-infective, " immune " mixture but to a lesser extent than of blood-cells obtained from a " normal " mixture, and they assumed that it indicated destruction of virus. A similar interpretation might have been put on the results of the present experiment,

TABLE II.—*Fate of Vaccinia Virus in a Mixture of Normal Leucocytes and Immune Serum; Fixation of Antiviral Substance by Leucocytes. Mixtures Incubated on Mechanical Shaker at 37° C. for 3½ Hours.*

Mixture.	Fraction tested.	Dilution.	Result.
Leucocytes in fresh plasma (1·2 c.c.) + normal serum (0·15 c.c.) + filtered vaccinia (0·15 c.c.)	Total	Undiluted	+++
	Washed leucocytes	10^{-2}	++
		10^{-3}	++
		10^{-4}	+
		10^{-5}	−
	Supernatant liquid	10^{-1}	+++
		10^{-2}	+±
		10^{-3}	+
		10^{-4}	−
Leucocytes in fresh plasma (1·2 c.c.) + immune serum (0·15 c.c.) + filtered vaccinia (0·15 c.c.)	Total	Undiluted	−
	Washed leucocytes	10^{-2}	+±
		10^{-3}	−
		10^{-4}	−
		10^{-5}	−
Leucocytes in fresh plasma (1·2 c.c.) + immune serum (0·15 c.c.). *No virus*	Washed leucocytes frozen and thawed 10 times + virus in indicated dilutions	$0{\cdot}5 \times 10^{-1}$	+
		10^{-2}	±
		10^{-3}	−
		10^{-4}	−
..	Virus control	10^{-2}	++
		10^{-3}	++
		10^{-4}	+
		10^{-5}	−

were it not for the fact that the " control " definitely indicated that leucocytes exposed to the same amount of immune serum and washed with the same amount of fluid, still contain sufficient protective substance to account for the observed difference in the titre. Since, as was previously pointed out, any immune serum loosely attached to the cells would have been diluted about 1 : 50,000, which is well beyond its effective range, one must assume that the leucocytes fixed the protective substance. In another experiment it was found, as would be expected, that leucocytes obtained from the blood of an immune animal also had fixed protective substance, but it could be shown that, as with minced testis, it was possible to remove it by profuse washing.

IS VACCINIA OPSONIZED BY IMMUNE SERUM?

Although the results of the preceding experiment failed to show an opsonic action on the virus, the evidence was indirect because of the fixation of protective substance by the leucocytes. It was therefore desirable to do an experiment in which such fixation might be eliminated as far as possible. The immune antibody which opsonizes bacteria combines with them, so that the opsonic effect is demonstrable with bacteria centrifuged from immune serum. An experiment along similar lines was planned with vaccinia. 1 c.c. of virus (Berkefeld "V" filtrate, with a titre of 1 : 10,000) was mixed with 0·5 c.c. of immune serum and 0·5 c.c. saline; a similar mixture was prepared with normal serum. After incubation at 37° C. for 1 hour and at room temperature for 18 hours, samples were removed from each tube, and the rest was centrifuged at 14,000 r.p.m. for three 1-hour periods. The supernatant liquids were pipetted off and discarded, while the sediments were resuspended in a volume of saline equal to that of the original virus. The leucocytes used in this test were derived from a 24-hour "aleuronat-starch-broth" pleural exudate, which was mixed with an equal volume of 1 : 1000 heparin in saline. One ampoule contained 1 c.c. of the leucocyte suspension and 0·5 c.c. of the reconstituted sediment from the mixture of normal serum and virus, and the other the same amounts of leucocyte suspension and reconstituted sediment from the mixture of immune serum and virus. After being shaken on the machine for 3½ hours at 37° C., the leucocyte mixtures were fractionated into supernatant liquid and cells by centrifugation. All the fractions were titrated on the same rabbit, and the results are shown in Table III.

TABLE III.—*Is Vaccinia Virus Opsonized by Immune Serum?*

Fraction tested.		Dilution.	Result. Normal serum.	Result. Immune serum.
Total serum-virus mixture after incubation for 1 hour at 37° C. and 18 hours at 16° C., before ultra-centrifugation.		Undiluted	++++	−
Reconstituted sediment after ultracentrifugation		10^{-1}	+++±	+++
		10^{-2}	++±	++
		10^{-3}	+	+
		10^{-4}	+	+
		10^{-5}	−	−
Ultracentrifuged sediment + normal leucocytes incubated at 37° C. for 3½ hours	Supernatant liquid	Undiluted	+++±	++
		10^{-1}	++±	+
		10^{-2}	+	−
	Leucocytes	10^{-2}	+++	++
		10^{-3}	++±	++
		10^{-4}	+±	+
		10^{-5}	−	−

The tests on the serum-virus mixtures confirmed results obtained in the first investigation (Sabin, 1935*a*), in that the sediment obtained from the "immune", innocuous mixture was quantitatively as infective as that obtained from the "normal" mixture. The results with the leucocyte fractions indicated that the virus centrifuged from the immune serum mixture was not opsonized. The leucocytes from each of the reconstituted sediment mixtures were equally infective.

FATE OF VACCINIA VIRUS AFTER INTRAVENOUS INOCULATION INTO AN IMMUNE RABBIT.

It has been demonstrated by Smith (1929), and confirmed by Long and Olitsky (1930), that when vaccinia is injected intravenously into an immune rabbit, the washed blood-cells obtained, even 4 hours later, are infective, while the whole blood is not. This experiment in itself suggests that the blood-cells do not destroy the virus even *in vivo*, and that the animal must be protected from infection by another mechanism. Smith (1929) observed, however, that blood-cells obtained similarly 6 hours or more after inoculation were not infective, and the question naturally arises whether the virus is destroyed in the cells by that time, or whether most of the cells bearing the virus are deposited in the liver, spleen and elsewhere. To determine whether the blood-cells are capable of destroying the virus after more prolonged fixation, it was proposed to repeat Smith's experiment, with the modification that the blood obtained 3 hours after inoculation would be incubated *in vitro* at 37° C. on a shaking machine for an additional 20 hours before fractionation, and the presence of virus in these cells compared with that in the cells obtained from the 24-hour bleeding of the same animal.

A vaccinia-convalescent rabbit was injected intravenously with 10 c.c. of Berkefeld "V" filtered virus. Three hours later 15 c.c. of blood was obtained in heparin (final dilution of heparin 1 : 2000) by cardiac puncture. A sample of the whole blood was saved and half of the remainder was sealed in an ampoule and put on the shaking machine at 37° C., while the rest was centrifuged at 3000 r.p.m. for 15 minutes. Whether it was because the available heparin was of poor quality or for other reasons, clotting could not be avoided entirely; in this case the buffy layer was clotted, while the plasma and red cells were fluid. Since it was found in other experiments, not here recorded, that the clot contained the virus, the thin, clotted, buffy disc was removed, washed 3 times in 10 c.c. amounts of saline, and finally ground with sand and 1 c.c. of saline. Since it was desirable to test all the fractions on the same rabbit, this fraction was put in the cold room until the next day. Twenty-four hours after inoculation another 10 c.c. of blood was obtained by cardiac puncture and centrifuged; the buffy layer, which was again clotted, was washed and ground with sand in the same manner. The 3-hour blood-sample which was incubated *in vitro* was treated the same way. 0·2 c.c. amounts of the various fractions were injected intracutaneously in the same rabbit. Quantitative determinations would have been significant only if all the blood-cells from a given volume were titrated, but since adequate washing of a large volume of blood-cells is difficult, and in view of the clotting, it was not undertaken.

The results shown in Table IV indicate that whereas the animal was adequately immune, since both the 3-hour and 24-hour total blood samples were not infectious, the washed clots obtained from them as well as from the 3-hour blood, which was incubated *in vitro*, contained active virus.

TABLE IV.—*Fate of Vaccinia after Intravenous Inoculation into an Immune Rabbit.*

Bleeding.	Fraction tested for virus.	Result.
3 hours after inoculation	Whole blood stored at 1° C. for 20 hours	—
	Washed cells	++
	Whole blood shaken at 37° C. for 20 hours; washed cells tested	++±
24 hours after inoculation	Whole blood	—
	Washed cells	++±

DISCUSSION.

The main purpose of the present investigation was to determine whether leucocytes were capable of destroying vaccinia virus, and whether or not anti-vaccinial serum could exert an opsonic effect on the virus. To begin with, fresh normal rabbit serum and leucocytes were not found to be viricidal, contrary to the experience of Douglas and Smith (1930). It was observed, however, that the leucocytes fix the virus in much the same manner as minced testis does, but that they remain highly infectious in the intact state, even after 20 hours' incubation at 37° C. In the experiments in which immune serum was used, special attention was paid to the adequate washing of the cells following incubation. When leucocytes, immune serum and virus were incubated together, it was found that although the total mixture was not infective, the washed cells obtained from it contained active virus. The amount of virus demonstrable on such cells was, however, less than on cells obtained in a similar manner from a normal serum mixture. These results are similar to those obtained by Douglas and Smith (1930), but whereas they interpreted them as indicating destruction of virus due to an opsonic effect of the serum, it could be shown in a simultaneous control experiment that leucocytes treated with the same amount of immune serum, and washed in a similar manner, had fixed sufficient protective substance to account for the observed quantitative difference. This interpretation was further confirmed when it was found that virus which had been incubated with either normal or immune serum, and subsequently ultracentrifuged, was fixed apparently to the same extent by leucocytes, which then became equally infectious. It was thus clear that *in vitro* the leucocytes do not destroy vaccinia in the presence of immune serum. That such destruction by leucocytes also fails to occur *in vivo* was evident from the fact that even 24 hours after intravenous injection of vaccinia into an immune rabbit, virus was still demonstrable in the washed cells, while the whole blood was not infective.

In the preceding investigations it was shown that the tissue cells are protected by immune serum without any associated destruction of virus, and in

the present investigation the evidence is that it is not destroyed by the leucocytes. It is quite probable that active virus may survive in the immune animal for a long time, as is, indeed, suggested by the results of Douglas, Smith and Price (1929), who recovered vaccinia from the ovary, testicle, adrenal, tongue and brain of rabbits 41 days after infection, and by those of Olitsky and Long (1929), who by cataphoresis demonstrated vaccinia in immune animals several months after infection; the virus, however, is innocuous for the immune animal because the tissues are protected.

In an abstract of a preliminary report by Beard and Rous (1934), it is stated that normal Kupffer cells and clasmatocytes are capable of "neutralizing" vaccinia while leucocytes are not. In a personal communication Dr. Beard kindly pointed out that this "neutralization" represents a modification of the vaccinial lesion as regards size and form, and that this effect is exerted by cells from normal animals and in the absence of immune serum. Whether or not Kupffer cells and clasmatocytes are capable of destroying vaccinia is not known, however, but further investigation should reveal what part they play in the immune process.

SUMMARY.

1. Fresh normal rabbit serum and leucocytes are not viricidal for vaccinia.

2. In the presence of normal serum leucocytes "fix" the virus, but become and remain highly infectious thereby.

3. Leucocytes also fix virus in the presence of immune serum, but no evidence was obtained that it was subsequently destroyed.

4. Leucocytes can fix the protective substance in immune serum in much the same manner as minced testis.

5. Virus incubated with immune serum and ultracentrifuged is fixed apparently to the same extent, and the leucocytes remain as highly infectious as when virus which has been ultracentrifuged after incubation with normal serum is used.

6. While the whole blood of an immune rabbit, injected intravenously with vaccinia, is not infectious, the leucocytes were shown to contain active virus even 24 hours after inoculation.

7. There is, therefore, no evidence that the immune serum exerts an opsonic action on vaccinia, or that the leucocytes can destroy the virus in its presence.

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THE MECHANISM OF IMMUNITY TO FILTERABLE VIRUSES. IV. THE NATURE OF THE VARYING PROTECTIVE CAPACITY OF ANTIVIRAL SERUM IN DIFFERENT TISSUES OF THE SAME SPECIES AND IN THE SAME TISSUES OF DIFFERENT SPECIES.

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Received for publication February 25th, 1935.

A PROPER understanding of the phenomenon first described by Andrewes (1928) and Todd (1928) is essential to the elucidation of the mechanism of immunity to filterable viruses. Andrewes (1928) observed that mixtures of vaccinia and immune serum which were innocuous for the skin were nevertheless infective when injected intratesticularly, intracerebrally or intravenously, and that skin-inactive mixtures of Virus III and the corresponding immune serum were likewise infective when injected intratesticularly or intravenously. Todd (1928) found that mixtures of fowl-plague virus and immune serum, which were inactive when injected intramuscularly, were infective when injected intravenously. Recently the same phenomenon was observed with B virus immune serum, which failed to prevent infection when injected with minimal quantities of the virus intracerebrally, while it protected very well by the intracutaneous route (Sabin, 1934). A corollary to this phenomenon of the varying protective capacity of antiviral serum in different tissues of the same species may be found in Shope's (1931) observation with pseudorabies-immune serum, the protective power of which varies markedly in the same tissue (subcutaneous) of different species (guinea-pig and rabbit). All these observations raise many questions, the answers to which would go a long way in giving a clearer insight into the operation of the mechanism of immunity. Does this phenomenon merely depend on the fact that smaller amounts of free virus may be detected by different routes of inoculation ? There is already some evidence that this is probably not the case ; Todd (1928) found that the intramuscular route is quite as sensitive, and probably even more so, than the intravenous route for detecting small amounts of fowl-plague ; in some of Andrewes' (1928) experiments with vaccinia and Virus III, and in the experiments with B virus (Sabin, 1934), it was found impossible to secure protection against minimal infective amounts of virus. Is this phenomenon in agreement with the conception that immune serum inactivates the virus *in vitro ?* It is difficult to see how the virus could be inactivated *in vitro* and yet still be infectious when inoculated by certain routes. Fairbrother (1932) claimed, however, that " contact *in vitro* between immune serum and vaccinia virus plays an important *rôle* in determining the infectivity of the mixture on intracerebral inoculation ", but this conclusion

is based on a single experiment, in which a few rabbits failed to die after developing signs of encephalitis, and is not confirmed by data presented in a subsequent report (Fairbrother, 1933). Furthermore, in some of the experiments on vaccinia and Virus III (Andrewes, 1928) and on B virus (Sabin, 1934), the mixtures which were inactive by one route and infectious by another were incubated for relatively long periods of time. If one assumes, however, as the data presented in the preceding communications (Sabin, 1935*a*, 1935*b*, 1935*c*) lead one to suspect, that the protective action of the immune serum is in some way bound up with the tissue cells, what are the factors which enable the same serum to protect one tissue and not another? Is it possible that infection may occur in some tissues and not in others because in a mixture of the two the virus is "fixed" before the protective substance, or because the protective substance is washed away (by diffusion) more readily than the virus, or because some cells require more protective substance than others to be rendered refractory to infection with the same amount of virus?

The purpose of the present investigation was to determine (*a*) the relation between the minimal infective dose of a virus when administered by different routes and the protective capacity of immune serum by those routes, (*b*) the effect of increasing the amount of antiviral serum and decreasing the amount of virus on the infectivity of mixtures by different routes, and (*c*) the effect on infectivity of allowing the antiviral serum to come in contact with the tissues before the virus is introduced.

RELATION BETWEEN THE MINIMAL INFECTIVE DOSE OF VACCINIA AND THE PROTECTIVE CAPACITY OF ANTIVACCINIAL SERUM BY THE INTRACUTANEOUS AND INTRACEREBRAL ROUTES.

The vaccinia virus used in these experiments was a "neuro-testicular" strain in the form of a Berkfeld "V" filtrate of a 5 p.c. suspension. Ten-fold dilutions in saline, prepared with separate pipettes, were tested by injecting 0·2 c.c. amounts intracutaneously or intracerebrally. The final intracutaneous titre was recorded on the 6th day, or earlier if generalization occurred before that time. For the determination of the intracerebral titre, daily temperatures were taken and observations made on the inoculated rabbits; all the recovered rabbits, whether or not they developed fever and other clinical signs of infection, were tested for active immunity 15 to 21 days after inoculation by the intracutaneous injection of approximately 1000 and 100,000 M.I.D. The last rabbit to show immunity was considered to have had a minimal infective amount of virus. By this method it was found that the minimal infective dose of vaccinia intracerebrally was roughly the same as, or within a ten-fold dilution less than, by the intracutaneous route. Dr. E. W. Hurst (personal communication), who was performing similar titrations for another purpose, obtained similar results. It should be noted that rabbits injected intracerebrally with vaccinia, particularly with small doses, do not react uniformly; some respond only with fever and subsequent immunity to a dose of virus ten times that which may kill others. Generally it was found that rabbits which had fever were subsequently immune, while those that had no fever

were not. It was apparent from these tests that the infectivity for the brain of serum-virus mixtures innocuous for the skin could not be attributed to a greater sensitivity for vaccinia.

Although there was no good evidence in reported work that a serum-virus mixture, innocuous for the skin and infective for the brain, could be rendered non-infective by prolonged incubation, it was desirable to determine this directly in the case of vaccinia with a dose of virus similar to that which Fairbrother (1932) used in his experiments. 0·1 c.c. of the antivaccinial serum used in this test protected against 100,000 M.I.D. of the virus by way of the skin. A mixture of equal amounts of filtered virus, containing 1000 M.I.D. in 0·1 c.c., and immune serum was incubated at 37° C. for 4 hours and in the cold room for 14 days. At the end of that time, 0·2 c.c. was injected intracutaneously in one rabbit, and 0·2 c.c. intracerebrally into another. While no lesion developed in the intracutaneously injected rabbit, the rabbit injected intracerebrally showed signs on the 5th day, was prostrate and had convulsions on the 6th day, remained alive, prostrate and cold for 5 more days, and died on the 12th day after inoculation. It was obvious from this test that although the mixture was innocuous for the skin, the virus was not inactivated even after 14 days' incubation, and that prolonged incubation was not a factor in determining the infectivity of a serum-virus mixture on intracerebral inoculation.

The purpose of the next experiment was to determine whether by increasing the amount of immune serum in relation to virus one could modify the infection in the brain, and whether or not infection could be entirely prevented when a minimal dose of virus was used. The same antivaccinial serum was used, 0·1 c.c. of it protecting against at least 100,000 M.I.D. by the intracutaneous route in several rabbits. The virus was a Berkfeld "V" filtrate, and was titrated simultaneously with this test by both the intracutaneous and intracerebral routes in rabbits. Mixtures of diluted virus and varying amounts of immune serum were prepared, and after about one hour at room temperature injected intracerebrally in rabbits. The results are shown in Table I. It may be seen that the M.I.D. of this virus preparation by way of the brain was a lesser dilution than 1 : 50,000 for 0·1 c.c., and probably 1 : 5000, corresponding roughly to the intracutaneous titre obtained on one rabbit and perhaps 10 times less than that on two others. The rabbits receiving 0·1 c.c. of the 1 : 50 dilution (approximately 100 M.I.D.) mixed with either 0·1 c.c. or 0·2 c.c. of the serum died with signs of meningo-encephalitis, while the one receiving the same amount of virus with 0·4 c.c. of serum survived; but this survival does not indicate that infection was prevented, since the rabbit had fever for several days and was subsequently immune. Similarly, the rabbits which received 0·1 c.c. of the 1 : 500 dilution (approximately 10 M.I.D.) and 0·1 c.c. of the 1 : 5000 dilution (about 1 M.I.D.) with 0·4 c.c. of serum, while they survived, showed fever and subsequent immunity, indicating that infection was not prevented. Although 0·1 c.c. of this serum diluted 1 : 100 was still capable of preventing a skin lesion with 1 M.I.D. of virus, apparently 400 times as much was incapable of preventing infection by way of the brain. It is almost impossible to correlate such findings with any view which presumes destruction of the virus by the immune serum, either *in vitro* or *in vivo*.

TABLE I.—*Effect of Antivaccinial Serum by the Intracerebral Route.*

0·1 c.c. of the serum used in this test protected against 100,000 minimal infective doses by the intracutaneous route.

Virus.		Amount of serum (c.c.).	Result.			
Amount.	Dilution.		Fever.	Signs.	Survival.	Subsequent immunity.
0·1 c.c.	$0·2 \times 10^{-1}$	0·1	7, 8*	9*	D. 11†	..
,,	,,	0·2	4, 5, 6, 7	7	D. 11	..
,,	,,	0·4	4, 5, 6, 7, 8	None	Survived	Immune.
,,	$0·2 \times 10^{-2}$	0·4	5, 6, 7, 8	,,	,,	,,
,,	$0·2 \times 10^{-3}$	0·4	2, 3, 4, 5	,,	,,	,,
,,	$0·2 \times 10^{-4}$	0·4	None	,,	,,	Not immune.
,,	$0·2 \times 10^{-4}$‡	None	,,	,,	,,	,,
,,	$0·2 \times 10^{-5}$	,,	,,	,,	,,	,,
,,	$0·2 \times 10^{-6}$	,,	,,	,,	,,	,,

* The numbers under "fever" and "signs" refer to days after inoculation.

† D. 11 = dead 11 days after inoculation.

‡ The same virus suspension was titrated intracutaneously in three rabbits; in two the last positive reaction was with 0·2 c.c. of 1 : 100,000, and in the other with 0·2 c.c. of 1 : 10,000 dilution.

COMPARATIVE EFFECT OF ANTIHERPES SERUM BY THE INTRACUTANEOUS AND INTRACEREBRAL ROUTES.

The data on a comparative titration of a potent antiherpes serum against known amounts of virus (Perdrau EL_1) by both the intracutaneous and intracerebral routes are shown in Table II. It may be seen that whereas 0·002 c.c. of the serum was sufficient to protect against 50, < 500, M.I.D. of virus by way of the skin, 0·1 c.c., or 50 times as much serum, had no effect on the same number of minimal infective doses by way of the brain. The mixtures were incubated for 4 hours at room temperature before inoculation. The rabbits injected with 10, < 100 intracutaneously and 1, < 10 M.I.D. by way of the brain with 0·1 c.c. of the serum survived. Unfortunately, no tests for immunity were made on these rabbits, and it is difficult to say whether or not they had completely escaped infection. It is not improbable, however, that antiherpes sera, like antipoliomyelitis sera, may have a definite effect in the brain, although apparently to a considerably lesser degree than in the skin.

TABLE II.—*Effect of Antiherpes Serum by the Intracutaneous and Intracerebral Routes.*

Route.	M.I.D. of virus.	Amount of serum (c.c.).	Result.
Intracutaneous	50, <500	0·001	Herpetic lesion.
	,,	0·002	No lesion.
	,,	0·02	,,
	,,	0·1	,,
Intracerebral	50, <500	0·02	D. 5.*
	,,	0·1	D. 5.
	10, <100	0·1	Survived.
	1, <10	0·1	,,

* D. 5 = dead 5 days after inoculation, with signs of encephalitis.

COMPARATIVE EFFECT OF ANTI-B VIRUS SERUM BY THE INTRACUTANEOUS AND INTRACEREBRAL ROUTES.

It has already been shown (Sabin, 1934) that the M.I.D. of the B virus by way of the skin is practically the same as that by way of the brain, and also that a serum which protects very well by the intracutaneous route fails to prevent infection and death with even a minimal dose of virus by the intracerebral route, in spite of a preliminary 24 hours' incubation. In casual tests it was observed, however, that occasionally a rabbit injected with a serum-virus mixture failed to be infected, and that a repeated inoculation of the same serum-virus mixture into another rabbit killed promptly. It appeared that either one was dealing with amounts of serum which were on the threshold of effectiveness, or that in the cases of survival the mixtures might perhaps have been injected intraventricularly or intrathecally instead of intracerebrally. Whether the effect of a serum-virus mixture, when in contact with meningeal or ependymal cells, may be different from that which follows contact with brain-cells is not known, and was not established in these experiments.

The purpose of the following experiment, however, was to determine whether, by taking special precautions that the injection is in the brain substance itself (mainly by making all injections at the same site through a hole in the cranium, using a short needle and inserting it the same distance and by aspirating before injection), and by increasing the amount of serum, it would be possible to show a protective effect against small, but constantly infective, amounts of virus. It should be stated that with the B virus in rabbits there are no abortive or unrecognized infections leading to immunity; the smallest effective amount of virus by any route is fatal.

TABLE III.—*Action of Anti-B Virus Serum by the Intracutaneous and Intracerebral Routes.*

Experiment.	Route.	Amount of 10 p.c. virus suspension (c.c.).	Amount of serum.	Result.
I	Intracutaneous	0·002	None	S. 3, Par. 8, D. 9.*
		0·0002	,,	S.
		0·00002	,,	S.
	Intracerebral	0·002	,,	D. 4.†
		0·0002	,,	S.
		0·00002	,,	S.
	Intracutaneous	0·1	0·1	S.
		0·1	0·02	S.
		0·1	0·004	S.
		0·1	0·002	S.?, Par. 9, D. 11.
	Intracerebral	0·1	0·1	D. 4.
		0·02	0·1	D. 4.
		0·002	0·1	D. 5.
II	Intracutaneous	0·05	0·05	S.
	Intracerebral	0·05	0·05	D. 4.
		0·05	0·15	D. 5.
		0·05	0·30	D. 5.
		0·05	0·45	D. 5.
III	Intracutaneous	0·002	None	S. 4, Par. 8, D. 9.
		0·0002	,,	S. 4, Par. 8, D. 9.
	Intracerebral	0·005	,,	D. 5.
		0·0005	,,	D. 6.
		0·00005	,,	S.
		0·000005	,,	S.
	,,	0·005	0·05	D. 5.
		0·005	0·15	Par. 6, D. 7.
		0·005	0·45	Par. 8, D. 11.
	,,	0·0005	0·45	D. 10.
		0·00005	0·45	S.

* S. 3, Par. 8, D. 9 = skin lesion 3rd day, paralysis 8th day, dead 9th day after inoculation.
† D. 4 = dead 4th day after inoculation with signs of encephalitis.

The results are shown in Table III. The comparative determinations of the M.I.D. which were performed simultaneously again show that the rabbit is equally susceptible to the virus introduced by way of the skin or brain. It may also be seen that whereas 0·004 c.c. of the immune serum protected against 0·1 c.c. of a 10 p.c. suspension of the virus (*i. e.* 50 to 500 M.I.D.) by the intracutaneous route, 0·45 c.c., or more than 100 times as much of the same serum, failed to protect against 0·0005 c.c. of the virus—approximately a minimal dose—by the intracerebral route. Whether or not it was due to the special precautions which were taken, not a single one of the eleven rabbits inoculated with immune serum and an infective amount of virus survived. It is, perhaps, interesting to note that in three rabbits, which were inoculated intracerebrally with small amounts of virus and relatively large amounts of serum, there was apparently some modification in the course of the disease, consisting in one of a prolongation of the incubation period, and in the two others of a primary development of flaccid paralysis of the posterior extremities and a rather longer survival.

EFFECT OF ANTI-PSEUDORABIES SERUM IN RABBITS AND GUINEA-PIGS BY THE SUBCUTANEOUS ROUTE.

In the preceding experiments, the phenomenon of the varying protective capacity of antiviral sera in different tissues of the same species was shown not to depend on a variation in susceptibility to the virus of the different tissues, but rather to special, as yet unknown, conditions obtaining in them. In the case of pseudorabies, the effectiveness of the antiviral serum appears to vary in the same tissue of different species, which, however, are not equally susceptible to the virus. It was important to determine, therefore, whether in the case of pseudorabies this phenomenon (Andrewes-Todd) could be accounted for on the basis of the greater susceptibility of the rabbit to the virus. When Shope (1931) first tested anti-pseudorabies serum in rabbits and guinea-pigs it appeared as if the serum could protect guinea-pigs but not rabbits by the subcutaneous route, but he stated that later tests revealed that rabbits could also be protected when mixtures containing larger amounts of immune serum and smaller doses of virus were used. Mixtures which were inactive for guinea-pigs by the subcutaneous route were also inactive when injected intraperitoneally, and preliminary incubation of the serum-virus mixtures was not found to be necessary.

An amount of virus which constitutes 1 M.I.D. for the guinea-pig by the subcutaneous route is roughly 10 and sometimes 100 M.I.D. for the rabbit by the same route. If immune serum inactivated virus *in vitro*, one would expect that if one decreased the amount of virus by 100 or 500 M.I.D. in a mixture which is infective for rabbits but not for guinea-pigs, it would then also become non-infective for rabbits; at least one would expect it to be the case if the phenomenon in question depends on the fact that smaller quantities of virus can be detected by using the rabbit. Actually, however, this did not prove to be the case. The pseudorabies virus consisted of a 10 p.c. suspension of glycerolated rabbit brain in saline, centrifuged at about 2000 r.p.m. for 10 minutes. The anti-pseudorabies serum (immune swine) was obtained through

the courtesy of Dr. R. E. Shope, of the Rockefeller Institute, Princeton, N.J. The virus and serum were mixed *in vitro*, and were injected sometimes after a few hours at room temperature, and at other times after additional incubation in the cold room overnight. The same mixtures were injected simultaneously in rabbits and guinea-pigs. The results are shown in Table IV.

TABLE IV.—*Effect of Anti-pseudorabies Serum in Rabbits and Guinea-pigs by the Subcutaneous Route.*

Experiment.	Amount of 10 p.c. virus suspension (c.c.).	Amount of serum (c.c.).	Effect in rabbit.	Effect in guinea-pig.
I	1·0	0·02	D. 72*	D. 92.
	1·0	0·10	D. 72	D. 72
	1·0	0·20	D. 92	Survived.
	0·2	0·10	D. 92	,,
	0·2	0·20	D. 120	,,
II	1·0	0·10	..	Survived.
	1·0	0·20	D. 72	..
	1·0	0·50	D. 96	..
	1·0	1·00	D. 96	..
	0·2	0·60	Survived	..
	0·1	0·10	,,	..
	0·1	0·20	,,	..
	0·1	0·40	,,	..
	0·1	0·80	,,	..
	0·1	None	..	D. 92.
	0·01	,,	..	D. 96.
	0·001	,,	D. 96	Survived.
	0·0001	,,	Survived	..
III	1·0	0·1	..	Survived.
	1·0	1·0	D. 110	..
	1·0	2·0	D. 136	..
	1·0	3·0	D. 136	..
	0·5	1·0	D. 96	..
	0·5	2·0	D. 164	..
	0·2	0·1	D. 136	..
	0·2	0·2	D. 164	..
	0·2	0·4	D. 280	..
	0·1	0·1	D. 168†	..

* D. 72 = dead 72 hours after inoculation, with evidence of pseudorabies.
† Did not bite or scratch; died with atypical signs.

The virus used in the first experiment had recently undergone five brain-to-brain passages in rapid succession, and was more potent than usual, requiring 0·2 c.c. of the immune serum to protect against 1 c.c. of the 10 p.c. virus in guinea-pigs; as may be seen in the subsequent tests, the potency of the immune serum was such that 0·1 c.c. ordinarily protected against 1 c.c. (10 p.c.) of the virus in guinea-pigs. In rabbits, however, it was found that 3 c.c., or 30 times

as much serum, failed to protect against the same amount of virus, and even 2 c.c. of serum failed to prevent infection with half the amount of virus. When the amount of virus was reduced to about a tenth of the original quantity, even small amounts of serum sufficed to protect rabbits. It was thus apparent that the varying protective capacity of anti-pseudorabies serum in rabbits and guinea-pigs was not due to the fact that the rabbit was capable of indicating smaller amounts of uninactivated virus, but rather to other conditions in the subcutaneous tissue of the two species, which may be only indirectly related to their susceptibility. It appeared as if, in the presence of a certain number of minimal infective doses of virus, the rabbit could not be protected, regardless of the amount of immune serum. It is obviously very difficult, if not impossible, to correlate such results with any hypothesis which supposes that the virus is inactivated *in vitro*.

COMPARATIVE EFFECT OF ANTI-PSEUDORABIES SERUM BY THE INTRANASAL AND INTRACEREBRAL ROUTES IN GUINEA-PIGS.

The M.I.D. of pseudorabies injected intracerebrally in guinea-pigs is approximately equal to that of pseudorabies injected subcutaneously in rabbits. It was therefore interesting to determine whether an amount of serum which is sufficient to protect rabbits by the subcutaneous route against certain doses of virus would be effective in protecting guinea-pigs when the mixture was injected intracerebrally. Such an experiment was also of interest as possibly indicating to what extent diffusion plays a *rôle* in this phenomenon, for the opportunities of diffusion and dilution in the guinea-pig's brain are probably no greater than in the rabbit's subcutaneous tissue. The question of diffusion was approached in still another way. Shope (1931) has already noted that guinea-pigs are susceptible to infection with the "Iowa strain" of pseudorabies by the intranasal route. In a preliminary investigation, using the Aujeszky strain, it was found that by merely dropping the virus on the uninjured nasal mucosa, less was required to infect and kill guinea-pigs than by subcutaneous injection, and that the intranasal M.I.D. was of the same order of magnitude as the intracerebral M.I.D. for guinea-pigs and the subcutaneous M.I.D. for rabbits. It was therefore possible to determine whether the infectivity of serum-virus mixtures by the intranasal route in guinea-pigs would be of the order observed in the subcutaneous tissue of the guinea-pig or rabbit, or whether it would correspond more closely to the infectivity of the mixture by the intracerebral route.

The results shown in Table V were quite unexpected with regard to the non-infectivity by the nasal route of serum-virus mixtures containing large amounts of pseudorabies. The immune serum appears to be quite as effective by the nasal route as it is in mixtures injected subcutaneously in guinea-pigs. Many other tests on the effectiveness of immune serum by the nasal route have since been performed, and the results obtained in this experiment have been confirmed. Of the three guinea-pigs which were injected intracerebrally with mixtures containing small amounts of virus, the ones receiving 0·1 c.c. and 0·01 c.c. of 10 p.c. virus died, while the one injected with a mixture containing the same amount of immune serum and 0·05 c.c. virus (5 times

TABLE V.—*Comparative Effect of Anti-pseudorabies Serum by the Intranasal and Intracerebral Routes in Guinea-pigs.*

Route.	Amount of 10 p.c. virus (c.c.).	Amount of serum (c.c.).	Guinea-pig No.	Result.
Intranasal	0·1	None	174	D. 68.*
	0·02		175	D. 68.
	0·01		176	D. 72.
	0·001		177	D. 114.
	0·001		178	Survived.
	0·0001		179	,,
Intranasal	1·0	0·2	181	,,
	0·5	0·2	182	,,
	0·2	0·2	183	,,
Intracerebral	0·1	0·2	188	D. 90.
	0·05	0·2	192	Survived.
	0·01	0·2	193	D. 72.

* D. 68 = dead 68 hours after inoculation.

more virus than one of the others) survived. It is not improbable that survival of this guinea-pig is of the same nature as the occasional survival of rabbits injected intracerebrally with B virus mixtures; but it is apparent, nevertheless, that although the guinea-pig brain is as sensitive to pseudorabies as the rabbit subcutaneous tissue, it is not as readily protected by immune serum, while the nasal mucosa (or free nerve-endings) of guinea-pigs is much more susceptible of protection than either. The results of the present experiment bring out many points elucidating the phenomenon under investigation. It would seem to show that the varying infectivity of similar serum-virus mixtures, when administered by different routes and in different species, cannot be directly accounted for by (*a*) the greater sensitiveness to the virus of one route over that of another, (*b*) any assumption that virus is inactivated *in vitro*, (*c*) greater diffusion and dilution by one route than by another. It rather seems to suggest that the protective capacity of the immune serum is dependent primarily upon the type of tissue and cell which is to be protected. If the tissue at the site of inoculation is easily protected, the virus apparently does not spread to some other part of the body which is not protected. This, of course, does not mean that diffusion and dilution cannot play a part in influencing the infectivity of a serum-virus mixture, particularly when such a mixture is injected intravenously and the amount of susceptible tissue to be protected is greatly increased.

CAN A SUB-EFFECTIVE AMOUNT OF ANTI-PSEUDORABIES SERUM BE RENDERED EFFECTIVE BY INOCULATION BEFORE THE VIRUS?

It was interesting to determine whether the ease with which mixtures of immune serum and virus infect certain tissues may possibly be due to the fact that the virus is fixed more rapidly or in greater quantity than the immune serum when the two are presented simultaneously, the cell thus becoming

infected in spite of an excess of protective substance all around it. If that were so, and if the tissue were at all capable of being protected, then one would expect that it might perhaps be achieved if it were allowed to come in contact with immune serum before being exposed to the virus. Such questions are obviously investigated better in tissue-containing cultures than *in vivo*. It was attempted, however, by injecting a number of rabbits intracutaneously with anti-pseudorabies serum, and at varying intervals thereafter inoculating the virus into the same zone. The amount of serum which was used was sufficient to protect a guinea-pig by the subcutaneous route when mixed with five times as much virus as was used to test the rabbits ; it was not, however, sufficient to protect rabbits when injected subcutaneously with this amount of virus. The intervals between the injections of serum and virus were a few seconds and 4, 24 and 48 hours ; another rabbit was injected with twice as much serum and half the amount of virus to determine whether or not protection was possible by the intracutaneous route. The results, shown in Table VI, indicate that whereas the latter rabbit survived, suggesting that

TABLE VI.—*Effect of Preliminary Injection of a Subeffective Amount of Anti-pseudorabies Serum on the Infectivity of Virus Subsequently Inoculated into the same Skin Zone (Rabbit).*

Serum and virus were injected intracutaneously at the same site.

Amount of serum.	Amount of 10 p.c. virus suspension.	Interval between serum and virus.	Result.
0·1 c.c.	0·2 c.c.	48 hours	D. 92
,,	,,	24 ,,	D. 96.
,,	,,	4 ,,	D. 120.
,,	,,	Few seconds	D. 144.
0·2 c.c.	0·1 c.c.	,,	Survived.

protection is possible, all the other rabbits died. From the length of the incubation periods it may be inferred that there was probably more protective substance at the site immediately after inoculation and at 4 hours than at 24 or 48 hours. Although swine serum (that was the source of the immune serum) gives rise to an inflammatory reaction in the skin of rabbits, and so might have influenced the results, it is doubtful whether a sub-effective amount of serum is any more effective when given before the virus. This question deserves more careful study however.

DISCUSSION AND SUMMARY.

The evidence presented in this communication indicates that the varying infectivity of certain mixtures of immune serum and virus, when administered by different routes or in different animals, is not due to the fact that one route may be more sensitive than another in detecting small amounts of uninactivated virus, nor to the fact that the mixtures are not sufficiently incubated *in vitro*. It is apparent, rather, that no inactivation could have occurred *in vitro*, and that the infectivity of the mixture is conditioned to a great extent by the character of the tissue into which it is introduced. That the mere

anatomical structure of the site of inoculation, particularly as regards the opportunities for diffusion and dilution, is not the deciding factor is evident from many observations. When one injects a mixture of antivaccinial serum and testicular virus intracutaneously in a rabbit, the skin may be protected even against 100,000 M.I.D. of virus although the diffusion of the mixture is apparently quite marked and rapid because of the testicle extract; nevertheless the same amount of serum, or even a larger amount, will fail completely to protect against 1000 or 10,000 times less virus when injected intracerebrally. In the case of the B virus even more than 100 times the amount of serum required to protect against 50 to 500 M.I.D. by the intracutaneous route fails to protect against approximately a single minimal infective dose of the virus by the intracerebral route, although rabbits are equally susceptible to infection by both routes. With pseudorabies the importance of the character of the tissue rather than the opportunities for diffusion or the sensitivity of the route is even more apparent. The minimal effective dose of pseudorabies is roughly of the same order of magnitude by the intracerebral and intranasal routes in the guinea-pig and by the subcutaneous route in the rabbit; yet, an amount of virus which cannot be protected against with large amounts of serum (3 c.c.) when injected subcutaneously in rabbits, is readily protected against by even small amounts of serum (0·2 c.c.) when it is slowly dropped into the nares of a guinea-pig, though even 100 times less virus mixed with the same amount of serum is fatal when injected into the brain of a guinea-pig. In studying these data it is difficult to escape the conclusion which was arrived at in the in-vitro experiments of the preceding investigations (Sabin, 1935*a*, 1935*b*, 1935*c*), that the mechanism of immunity to these viruses is intimately bound up with the cells to be protected. One cannot readily correlate these findings with any hypothesis which assumes that protection is the result of the direct action of the immune serum on the virus. It appears to be quite definite that *in vivo*, given tissues of equal susceptibility and equal amounts of immune serum and virus, the outcome is not the same. The mechanism whereby one tissue is readily protected while another is not is quite obscure, and its elucidation is of the utmost importance to a clear understanding of the nature of immunity to filterable viruses.

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THE PREPARATION AND TESTING OF ELEMENTARY BODY SUSPENSIONS FROM VACCINIA FILTRATES AND THEIR POSSIBLE USE IN SMALLPOX PREVENTION.

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Received for publication February 27th, 1935.

ALTHOUGH it is unlikely that the relatively small numbers of bacteria present in ordinary lymph as used for vaccination purposes* play any harmful *rôle*, there has recently been exhibited a tendency to investigate the possibilities of bacteria-free sources of vaccinia virus for human vaccination. These investigations have been made possible largely as a result of researches into the cultivation of the filterable viruses in general, and of vaccinia virus in particular. Vaccinia virus may be cultivated with comparative ease either *in vitro* or *in vivo*, and large quantities of highly active, bacteria-free virus may be obtained in this way. Without entering into the difficult problem of the possible harmful effects of such pyogenic bacteria as survive the treatment of lymph during its preparation for human vaccination, it would seem obvious that bacteriological sterility is desirable.

Rivers (1931) has shown that vaccinia virus may be cultivated in the presence of chick-embryo tissue and Tyrode's solution, and that an enormous multiplication of the virus occurs. With the bacteria-free culture-virus thus obtained typical vaccinial reactions were produced in lower animals and in man, thereby rendering them refractory to subsequent inoculation with ordinary lymph. Gallardo (1934) considers this method ideal, especially since, according to the latest experiences of Rivers, the culture-virus survives well in a desiccated state. Coffey (1934) also investigated the possibilities of culture-virus as used by Rivers, and obtained the dermal strain for initiating the cultures from him. It was her experience that the potency of the virus decreased markedly after 32 generations in chicken embyro, and that testicular passage through rabbits was necessary to restore its activity. She found that when children were vaccinated with culture-virus characteristic primary reactions were developed, but the percentage of "takes" was lower than among children vaccinated with calf lymph. She considered that further study was necessary to determine a method of preparing the culture-virus for distribution so that it might equal calf lymph in efficacy.

Ch'en (1934) has also investigated the possibilities of chick-embryo culture-virus. The potency of the virus after each passage through tissue culture for more than 40 passages showed considerable fluctuation in titre after the fifth subculture, when it reached its maximum potency. After this it declined until

* Under the provisions of the Therapeutic Substances Act (1925), vaccine lymph may contain not more than 5000 organisms in 1 c.c. Living gas-producing anaërobic organisms and hæmolytic streptococci must be absent.

by the 25th transfer it had fallen to the titre of the original virus inoculum. A further fall in titre occurred thereafter, until at the 30th subculture it appeared to have acquired biological stability. Passage through minced testis restored the titre at once. The immunizing power of the virus in various stages in the series was tested by intracutaneous injection in rabbits, where it was found that the degree of immunity produced was not in relation to the titre of the virus used. The suggestion was made, based on the results of intracerebral tests on rabbits, that vaccine of low titre might possess advantages from the standpoint of preventing vaccinial encephalitis.

Successful cultivation of vaccine virus by in-vivo methods on the developing chorio-allantoic membrane of chick embryos has been described by Goodpasture, Woodruff and Buddingh (1931, 1932), following the method of Woodruff and Goodpasture (1931) in their study of fowl-pox. They have suggested that this method should be applied to the preparation of anti-smallpox vaccine on a large scale. Their experiments have recently been repeated by Nauck and Paschen (1933) and by Stevenson and Butler (1933). In a recent paper (Goodpasture and Buddingh, 1933) a description of results obtained in human volunteers shows that by the " scratch " method successful vaccinations occur with this type of vaccine material. Some of the virus used had been cultivated on chick membrane through 75 successive passages, and had been stored at refrigerator temperature for three months. As a general rule the reaction produced was typical, except that it was somewhat milder and tended to follow a slightly delayed course. Subsequent vaccination with calf lymph showed a typical immune response.

Recently they (Goodpasture and Buddingh, 1934) have made further tests with their culture-virus by carrying out 980 primary vaccinations on school-children varying in age from 5 years to 20. Of these 92·9 p.c. were successful. Amongst the unsuccessful were a number of coloured children, so that a proportion of the failures might be put down to the difficulty in detecting signs of a previous attack of smallpox. From their studies they consider this type of vaccine entirely suitable for human vaccination.

Until quite recently the evidence regarding the filterability of vaccinia has been curiously discrepant. Yaoi and Kasai (1929), however, obtained highly active filtrates through Berkefeld " V " candles by altering the pH of the medium. Green and Eagles (1931) repeated their experiments, but found that the virus was readily filterable through these filters without preliminary alterations in the pH either of the filter itself or the fluid to be filtered. The loss in potency as a result of filtration was small, and the filtrate bacteriologically sterile. Eagles and Ledingham (1932), using these filtrates, described a method whereby suspensions of the vaccinia elementary bodies of Paschen (1906) could be prepared with the aid of the high-speed centrifuge. The entire infectivity of a Berkefeld " V " filtrate was shown to be present in such a suspension after high-speed centrifugation, and by the use of appropriate staining methods, the suspension was seen to consist of enormous numbers of elementary bodies.

In these bacteria-free filtrates and the elementary-body suspensions prepared from them another source of bacteriologically sterile vaccine material has been made available. But before these suspensions could be considered

suitable for vaccination it was essential that their behaviour in cold storage should be investigated. That elementary bodies remain active at 37° C. for a considerable time was shown by Amies (1934), who found that when they were suspended in a simple broth medium they withstood incubator temperature for several weeks, while at room temperature their potency remained at a high level for a considerable period. When suspended in normal saline the potency of the suspensions was diminished. In view of these findings he suggested that these suspensions might find a place in vaccination practice.

In the following communication details are given of the preparation of these suspensions, their distribution in ampoules and their infective titres after periods of conservation in the cold.

METHOD OF PREPARING SUSPENSIONS.

Commercial lymph was thoroughly rubbed with a glass rod into the skin of a rabbit immediately after the back had been closely shaved. After two or three passages in this way from rabbit to rabbit a vigorous "take" was produced, which reached the vesicular stage on the fourth or fifth day after inoculation. The animal was killed by intravenous injection of air—a procedure which diminishes the amount of blood in the skin. The virus material was removed with a sharp scalpel by light scraping. About 2 g. of pulp may be obtained in this way from one large, fully-grown rabbit. Only rabbits in good condition and with uniformly white skins should be used. The pulp was ground with a little distilled water in a mortar as thoroughly as possible. Distilled water was then added to made a 5 p.c. suspension. The suspension was well mixed, and was then centrifugalized at 3000 to 4000 r.p.m. for 15 minutes to remove heavy material. The supernatant fluid was filtered through a new Berkefeld "V" candle and the filtrate centrifugalized at 13,000–14,000 r.p.m. in the ultra-centrifuge for 1 hour. This served to precipitate most of the virus. After removal of the supernatant fluid the deposit was ground *in situ* with the rounded, etched end of a sterile glass rod. This preliminary grinding of the moist deposit served to produce a fairly uniform suspension of the elementary bodies in the deposit when suspending fluid was added. 10 c.c. of plain broth was added to the deposit. After being thoroughly mixed with a fine capillary pipette the broth suspension was tested aërobically and anaërobically for contaminating organisms.

When suspended in 10 c.c. of broth the elementary bodies obtained in this way from about 2 g. of pulp usually gave a titre of from 10^{-6} to 10^{-8} by intradermal inoculation.

It is not necessary to revert to the use of lymph after the first adaptation to rabbits for further inoculations. It should be noted, however, that when either lymph or lapine is passaged rapidly through a series of rabbits, the contaminating bacteria increase in virulence and an inflammatory reaction of the skin is set up, which is rapid and acute and precedes the vaccinial reaction by 48 hours or more. The appearance bears a close resemblance to vaccinia. The reaction may be so acute as to cause death of the animal. Suspensions, prepared from pulp removed under such conditions are invariably of very low titre and very few elementary bodies can be demonstrated. These early

non-vaccinial reactions may be avoided by using for animal inoculations either filtrates or suspensions of elementary bodies. When these are used and thoroughly rubbed into the freshly-shaved skin, vigorous, typical " takes " are the rule. Scarification additional to that produced by shaving is unnecessary, and is, moreover, harmful.

A number of suspensions have been prepared, distributed in ampoules, stored in the cold-room and tested at intervals from 27 to 400 days. Several suspending fluids have been used: plain broth, phosphate buffer (pH 7·3), normal saline, and normal saline with the addition of 0·25 p.c. agar. Agar was added in view of the results obtained by McClean and Eagles (1931) in a study of the conservation of vaccinia virus cultivated *in vitro*, where it was found to exert a beneficial effect in prolonging the viability of the virus under anaërobic conditions. Agar had been advocated by Gins, Hackenthal and Kamentzewa (1920), who found that 50 p.c. glycerine-saline solution rapidly weakened the virus, but that 2–3 p.c. agar solution was satisfactory as a diluting agent. It has the added advantages of causing the virus to adhere to scarified areas during potency tests, and of keeping the elementary bodies in uniform suspension. Gins (1921) employed agar as a diluent in 0·2–0·3 p.c. concentration in glycerinated lymph to prevent sedimentation of the particles. With more fluid suspensions great care must be taken to prevent the material from running off scarified areas.

In some cases the suspensions were stored without further dilution, while in others they were diluted fifty times before storage. No preservatives or antiseptics were added.

RESULTS.

In Table I is shown the titre of a number of suspensions in various suspending fluids at the time of preparation, and after storage at cold-room temperature for periods varying from 27 to 400 days. In three instances the pooled deposits after ultra-centrifugation of a filtrate were divided into two equal portions, and suspended in broth and in saline plus agar or in broth and in normal saline, in order to show the effect of suspending medium alone on the keeping properties of the suspension. These are indicated by large brackets.

From these results it appears that the majority of suspensions in broth and in saline plus 0·25 p.c. agar survive well at cold-room temperature for long periods, in three instances over a year. In two instances the survival in broth was poor.

Since it is the general practice for vaccination to be carried out by the scarification method, a number of stored suspensions were tested on the same rabbit by intradermal and scarification technique. In the intradermal tests 0·2 c.c. was used, and in the scarification tests as nearly as possible the same amount was rubbed with a glass rod into lightly scarified areas of approximately equal size. The results are shown in Table II.

As was anticipated the suspensions gave typical reactions by scarification, but were generally of a slightly lower titre than by intradermal technique. Where agar was present the titre was the same, since, owing to the more viscous nature of the suspending medium, more virus had infected the areas than in the case of broth and saline.

TABLE I.—*Showing Comparison of Titre of a Number of Suspensions when Freshly Prepared and after Varying Periods of Storage at ± 4° C.*

Suspension.	Original titre.	Storage in days at −4° C.	Titre after storage.	Dilution of original suspension.
EBS broth	10^{-8}	400	10^{-5}	Undiluted.
EBS saline + 0·25 p.c. agar	10^{-8}	400	10^{-5}	,,
E8 broth	10^{-6}	367	10^{-3}	1 in 50
E7 ,,	10^{-5}	315	10^{-4}	,,
EBS ,,	10^{-5}	218	Undiluted	,,
EBS ,,	10^{-4}	218	10^{-1}	,,
EBS phos. buffer	10^{-5}	218	10^{-2}	,,
E10 broth	10^{-6}	204	10^{-4}	,,
EBS broth	10^{-6}	115	10^{-5}	,,
EBS saline	10^{-6}	115	10^{-3}	,,
EBS broth	10^{-6}	91	10^{-5}	,,
EBS saline + 0·25 p.c. agar	10^{-6}	91	10^{-5}	,,
EBS saline	10^{-6}	61	10^{-3}	,,
155 broth	10^{-7}	27	10^{-7}	,,

TABLE II.

Suspension.	Titre by intradermal inoculation.	Titre by scarification inoculation.	Period of storage at −4° C. of suspension.
EBS broth	10^{-5}	10^{-4}	400 days.
EBS saline + 0·25 p.c. agar	10^{-5}	10^{-5}	400 ,,
E7 broth	10^{-4}	10^{-3}	315 ,,
EBS broth	10^{-5}	10^{-4}	115 ,,
EBS saline	10^{-3}	10^{-1}	115 ,,
EBS broth	10^{-5}	10^{-4}	91 ,,
EBS saline + 0·25 p.c. agar	10^{-5}	10^{-5}	91 ,,
155 broth	10^{-7}	10^{-6}	27 ,,

In order to demonstrate that the reactions on the rabbit were in fact due to vaccinia, neutralization tests were carried out ; for this purpose dilutions of antivaccinial serum were used with 1000 M.I.D. of a suspension in broth which had been stored for 315 days in the cold room. Control tests with normal serum were highly positive while the immune serum tests showed complete neutralization. As an additional control of the vaccinial nature of the stored suspensions, two rabbits that had received numerous inoculations during the course of testing the suspensions were bled after 10 to 12 days and their sera tested, by the author in co-operation with Dr. Amies, for agglutinins with a known suspension of elementary bodies from vaccinia. Specific agglutination occurred, demonstrating that suspensions of elementary bodies after long periods of storing are capable of producing an immunity response in susceptible animals.

DISCUSSION.

Although recent researches on vaccinia virus have produced sources of bacteriologically sterile virus, their adaptation to human vaccination against smallpox must be regarded for some time to come as in a purely experimental stage. From the available data it would appear, however, that there is a growing opinion in favour of replacing lymph, which practically always contains a number of viable, pyogenic bacteria, with a highly potent vaccine material, free from bacteria, and capable of conferring an immunity at least equal to that of lymph.

Neuro-testicular vaccine, which can be obtained free from bacteria with ease, has been widely used in Spain and Holland, but it has not found general favour on account of the severity of the reaction set up at the site of inoculation. Dermal virus has now been prepared as a bacteria-free alternative to both ordinary lymph and neuro-testicular virus. This material is available in the form of culture virus, obtained either *in vitro* or *in vivo*, or as filtrates of virus material, or the suspensions of elementary bodies obtained from them by high-speed centrifugation. In so far as can be judged by a limited number of vaccinations on humans over a short period of time, culture virus would appear to be quite satisfactory. Until now suspensions of elementary bodies from vaccinia have not received any serious consideration from this point of view, and experimental data are still derived only from animal investigations.

In this communication the possibility of elementary-body suspensions as an alternative to other vaccine materials is advanced. These suspensions, prepared from dermal lapine, represent the virus in its purest form, and they survive for long periods without serious deterioration in infectivity when stored at cold-room temperature. Plain broth, or saline with the addition of a small amount of plain agar as a suspending medium, exert a markedly beneficial effect in conserving their potency. The suspensions possess the advantage over filtrates, which themselves keep extraordinarily well, of consisting of practically pure virus particles, the serum proteins from the pulp having been to a large extent eliminated.

The method of preparation is simple, and large quantities of highly active virus may be obtained under laboratory conditions and without access to animals other than rabbits.

The reactions produced by these suspensions of elementary bodies do not vary in any way from those obtained with ordinary lymph, as judged by animal tests. They are neutralized by vaccinia-immune sera, and call forth an immunity response in rabbits shown by agglutinin production, and by the fact that inoculated animals are later immune to an injection of lymph. This occurs whether the intradermal or scarification method of inoculation is used.

At present, practically all human vaccinations for smallpox are made into the scarified skin. There is some hesitation to adopt the intradermal method. This is partly due to the fact that it has not been investigated in the human subject well enough to prove its efficacy beyond question, although it is in common use in the laboratory. Gallardo (1934) draws attention to the well-known fact that by the method of cutaneous scarification it is not possible to judge accurately the amount of virus retained by the skin. He considers

the intradermal technique difficult, especially in infants, and more painful than other methods. In his opinion the inoculation tends in children to be in reality a subcutaneous one, and, where lymph is used, the reaction is excessively slow with relative frequency in complications. When bacteria-free virus, in this case a neuro-testicular strain of normal activity, is available, he considers that the subcutaneous route offers unquestionable advantages, and he predicts that it is probably the technique of the future. In this connection the work of Yaoi (1934) is of interest. The vaccine he used was purified by adsorption and elution, and had a strength of 1 in 1000 minimal eruptive doses as determined by intra-cutaneous injection in rabbits. Altogether 613 persons were vaccinated subcutaneously, using 0·3 c.c. as a dose for infants and 0·5 c.c. for adults. The series comprised primary and secondary vaccinations, as well as vaccinations on adults who had been vaccinated at least twice previously. Revaccination by the percutaneous route always gave negative reactions, and although the time period following the subcutaneous vaccinations was not sufficiently long to warrant a conclusive opinion on the duration of immunity, there was nothing to suggest that it would prove less durable than that elicited by ordinary percutaneous vaccination. No mention was made of the incidence of complications contra-indicating the use of the subcutaneous route.

Suspensions of elementary bodies, as here described, should lend themselves to vaccination whatever technique is employed, but would appear, by virtue of their sterility and purity, to be particularly suitable for intradermal or subcutaneous inoculations. The ultimate test, however, must be their behaviour in routine human vaccination, and it is proposed to investigate this when an opportunity occurs. It is proposed, also, to investigate their properties when prepared in the dry state.

CONCLUSIONS.

A method has been described whereby large quantities of vaccinia virus of high potency, consisting of suspensions of elementary bodies, may be obtained.

These suspensions are a valuable addition to the sources of bacteriologically sterile vaccine virus, and may possibly find a place in vaccination practice.

They produce typical vaccinia reactions in animals either by intradermal inoculation or by scarification. These reactions are followed by immunity to subsequent vaccination.

The behaviour of these suspensions when used for human vaccinations will be investigated as opportunity offers.

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THE CULTIVATION OF VACCINIA VIRUS: FURTHER EXPERIMENTS WITH CELL-FREE MEDIUM.

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Received for publication February 27th, 1935.

Since the first report of successful culture of vaccinia virus in a medium free from whole living cells (Eagles and McClean, 1931), there have appeared communications by various investigators who have failed to obtain similar results. Only in two instances, however, are these described in sufficient detail to make evaluation possible. Maitland, Laing and Lyth (1932) were unable to carry the virus in subculture in the absence of living tissue. The experiments were made in parallel with those in which living tissue was used. They found it quite easy to initiate and maintain virus growth in a series of subcultures with living tissues, but beyond registering their inability to secure survival of the virus in the primary cell-free medium, they made no intensive effort to establish culture in series by concentrating on the problem of accommodation. Rivers and Ward (1933*a* and *b*) also attempted to cultivate vaccinia virus in lifeless media, using extracts prepared from minced rabbit kidney and testicle and chick embryos. They explained that their efforts were in part frustrated by the failure of Eagles and his co-workers (Eagles and McClean, 1930, 1931 ; and Eagles and Kordi, 1932) to provide an adequate description of their methods. In particular they found difficulty in interpreting the quantity of ingredients used, and the manner in which fresh rabbit serum and fresh testicular virus were freed from cells.

It was necessary, because of these failures, to re-investigate the whole

subject of the possibility of cultivating vaccinia virus in the absence of living tissue, and to determine if possible any sources of error either in technique or interpretation of our results. The present communication is an attempt to do this.

EXPERIMENTAL METHODS.

Preparation of Fresh Rabbit Serum.

A fully-grown rabbit was bled by cardiac puncture under ether anæsthesia, and the blood defibrinated by shaking with glass beads under sterile conditions. After centrifugation at 3000 to 4000 r.p.m. for half an hour the serum was removed and re-centrifugalized for the same period. The supernatant serum was thereby secured completely free of blood-cells.

Preparation of Cell-Free "Extract".

This was essentially similar to the method described by Eagles and Kordi (1932). It varied from this in that freezing and thawing was omitted.

After the rabbit had been bled it was killed with ether and the kidneys removed aseptically through the dorsal route. These, after the fat and capsule and as much as possible of the pelvis had been removed, were passed through a tissue-mincer with a disc of fine-mesh silver gauze. One c.c. of 9 p.c. saline was added, and the tissue ground without addition of abrasive substances in a large mortar until the mass attained a creamy consistency free from obviously large pieces. Sufficient sterile distilled water was added to restore the saline concentration to normal, and the whole transferred to 10 c.c. hard glass centrifuge tubes. It was then spun in the high-speed centrifuge for half an hour at 10,000 r.p.m.

After this treatment the tissue deposit was found to consist of three layers. The bottom one, comprising more than two-thirds of the whole, was found, on examination, to consist of clumps of macerated tissue with very large quantities of apparently whole cells. Overlying this was a layer of much finer deposit, which microscopically contained large quantities of cell-fragments, cell-nuclei, many whole cells attached to fragments, and amorphous material which could not be identified. The topmost layer of less than 0·5 cm. in depth was whitish and creamy in appearance. Microscopically it consisted of amorphous material, probably chromatic granules from cytoplasm, possibly mutilated nuclei of cells, and cell-fragments. No completely undamaged cells could be identified with certainty.

Avoiding the floating film of fat, the supernatant fluid was withdrawn with a fine capillary pipette as slowly as possible, leaving about half a centimetre above the topmost layer of deposit. This supernatant fluid on microscopic examination appeared to consist of enormous amounts of amorphous material, probably chromatic granules similar to that found in the deposit. Certain small fragments were found, but it was impossible to identify them beyond question as cell structures.

It should be stated here that no attempt was made to use constant, measured amounts of kidney tissue. Both kidneys were used, and since these vary in weight and size considerably in different rabbits, the amount of "extract"

obtained varied from experiment to experiment, although hypertonic saline and distilled water were used in constant quantity. The whole amount available was used, since it seemed advisable to obtain a medium as rich in material set free from the cells by grinding as was possible. In order, then, to keep the dilution of the virus constant throughout a series of subcultures, it was necessary to vary the relative amounts of serum and Tyrode's solution. The proportion of Tyrode's solution in the complete medium was roughly about half.

Tyrode's Solution.

The formula has been given in an earlier paper (Eagles and McClean, 1929), and varies in certain respects from the original. It should be noted that the original formula has been used by most workers, including Rivers, and Maitland, and their co-workers. Cultivation experiments with minced living tissue have shown, however, that slight variations in the formula of Tyrode's solution are not important.

The Virus Inoculum.

Two strains have been used—neuro-testicular virus and a pure suspension of elementary bodies prepared from dermal lapine.

Neuro-testicular virus was obtained by mincing and grinding a 4-day infected testicle with sufficient saline to make a 5 p.c. suspension, spinning at 4000 r.p.m. to remove large particles and filtering the supernatant fluid through a Berkefeld "V" candle. This served to remove cells and ensure sterility.

The elementary body suspension was prepared by a slight modification of the technique described by Eagles and Ledingham (1932). A dermal strain of virus was rubbed thoroughly on the freshly-shaved back of a full-grown rabbit, no additional scarification being used. About the fifth day, when the reaction was in the early stage of vesiculation, the animal was killed and the lapine harvested by scraping with a sharp scalpel. The lapine was thoroughly ground in a mortar without abrasive material with a small amount of distilled water. Sufficient additional distilled water was added to make a 5 p.c. suspension, and the whole mixed and spun at about 6000 r.p.m. for 15 minutes. The supernatant fluid was passed through a Berkefeld "V" candle and the filtrate spun at 14,000 r.p.m. for 2 hours. After removing the supernatant fluid the deposit was thoroughly mixed with a sterile glass rod and taken up in saline. After further mixing it was again spun lightly for 5 minutes to remove obvious clumps, and the supernatant was then tested for sterility and titrated intradermally on rabbits. The whole operation was carried out in one day. About 10 c.c. of highly active suspension could be obtained in this way. After suitable staining it was seen to contain enormous quantities of elementary bodies.

Preparation of Cultures.

Kidney extract, fresh rabbit serum, virus and Tyrode's solution were mixed in a flask. The amount of virus used throughout the series was a constant quantity (1 c.c.). The amounts of the other ingredients of the medium varied

within a comparatively small range, depending upon the amount of kidney extract available. The dilution of the virus was, in all cases, 1 in 20. Two examples are given in order to make this point clear:

Culture A.		*Subculture A.*	
Kidney extract . .	6·0 c.c.	Kidney extract . .	5·0 c.c.
Serum	4·0 ,,	Serum	4·0 ,,
Virus	1·0 ,,	Virus	1·0 ,,
Tyrode's solution . .	9·0 ,,	Tyrode's solution . .	10·0 ,,

The culture was then distributed in 2 c.c. amounts into Carrel flasks and incubated at 37° C. One c.c. was tested for sterility in plain broth, which was streaked on agar after 48 hours' incubation.

The first culture in a series was titrated before incubation intradermally on a rabbit, and the resulting titre used as an indication of the amount of virus present. Pre-incubation titrations were not made in subsequent subcultures in a series, the behaviour of the virus being measured in terms of titration and dilution at each subculture. As a general rule, nine flasks were set up at each experiment. Three flasks were titrated separately each day after 3 to 6 days' incubation. Occasionally flasks were tested after longer periods. Flasks were not pooled, because experience of the wide variations in virus content amongst individual flasks had shown that such a procedure is unwise. Each flask was tested for sterility.

In view of the considerable variation amongst rabbits in their reaction to vaccinia virus, a control intradermal injection of 0·2 c.c. of a 1 : 1000 dilution of commercial lymph was given to all animals used for titration at the time of injection of the culture dilutions. All the flasks titrated each day were tested on the same rabbit. Readings were recorded daily from the third to the seventh day following injection.

Technique of Titration.

A standard Dreyer dropping pipette was carefully calibrated from etched points to deliver 9 and 18 drops of saline when the pipette was held vertically. Eighteen drops of saline were run in this way into the requisite number of small tubes. Two drops of culture were added to the first tube, and thoroughly mixed by drawing up to the 9-drop mark and expelling. The operation was repeated a number of times, care being taken not to pass the mark. Two drops of this 1 in 10 dilution was added to the next tube and thoroughly mixed. The operation was repeated until the desired final dilution was reached. Before dilution of the next culture the pipette was attached to rubber tubing on the tap and thoroughly washed through. It was then washed with normal saline and as much moisture removed as possible. The same pipette was used for making the control lymph dilutions after making the culture dilutions. It was then again thoroughly washed in the manner described. Before using again to make culture dilutions, washing on the tap and with saline were repeated. For a short time washing with boiling water was used, but was discontinued because of the tendency to cause breakage.

The advantage in using the dropping pipette lay in that the same pipette

was in use throughout a series of subcultures, so that any error intrinsic in the method was constant. Varying degrees of pH which might be introduced by using large numbers of pipettes prepared in the ordinary way were avoided.

RESULTS.

Considerable difficulty was experienced, as in previous work, in initiating a successful series of subcultures. In the present series shown in Table I at least six cultures were carried out before sufficiently good survival of the virus was obtained to warrant subculture. It is apparently essential to secure good survival in the original culture in cell-free medium.

From these results it is apparent that large variations in titre occur amongst flasks, after the same period of incubation, throughout the series. There is a tendency for the titre to fall considerably in the first culture. In spite of the effects of dilution and periods of incubation on the original inoculum, some flasks show apparent multiplication. The fifth subculture was unsuccessful.

It was decided to attempt a new series with fresh neuro-testicular virus, and to ascertain at the same time the effect of subjecting the kidney extract to a second period of spinning at 10,000 r.p.m. In order to obtain sufficient extract four kidneys were used and the amounts of serum and Tyrode's solution doubled.

The results are shown in Table II.

The second series of subcultures with neuro-testicular virus was terminated at the seventh subculture in the case of the once-spun extract because no additional information was likely to be obtained by prolonging it. As in the first series, the amount of variation amongst flasks throughout was disconcerting. The short series using twice spun extract did not survive the fourth subculture.

It has been suggested that the strain of virus employed may be important in cultivation of vaccinia virus. It was decided, therefore, to attempt a series of subcultures, using dermal lapine instead of neuro-testicular virus. For this purpose a pure suspension of elementary bodies of high potency was used. The results of this series are outlined in Table III. As before, no attempt was made to carry on subculture indefinitely, and the series was terminated at a point where the presence of a large quantity of virus indicated multiplication in terms of the dilution factor.

There is no published report of persistent attempts to cultivate the virus of vaccinia in cell-free medium in which sufficient detail of the behaviour of the virus in individual flasks is given to enable an analysis to be made. A number of initial attempts at cultivation do not adequately provide this. Moreover, initial attempts, even in the presence of living tissue, are frequently unsuccessful. The three series of subcultures presented in this communication are typical of the experiences met with in cultivation in cell-free medium. The wide variations in titre found amongst flasks in a set of subcultures has been commented upon in earlier reports. The reason for this remains obscure.

It seemed advisable to investigate the possible sources of error in technique which might give a misleading impression that multiplication of virus had been obtained. In view, also, of the wide variations in titre amongst individual

TABLE I.—*Showing Series of Cell-free Subcultures using Filtrate of Neuro-testicular Virus as Seed.*

Subcultures.	Virus used and titre.	Titre of cultures and days of incubation.					
		3rd day.	4th day.	5th day.	6th day.	7th day.	8th day.
1st culture	Fresh N.T.V.; titre 10^{-5}	3 flasks 10^{-1} ..	2 flasks 10^{-1} 1 flask 10^{-2}	1 flask neg. 2 flasks 10^{-4}	..	..	..
1st subcult.	5th day cult.; titre 10^{-4}	3 flasks neg.	2 flasks 10^{-3} 1 flask 10^{-2}	2 flasks 10^{-4} 1 flask 10^{-1}	..	..	..
2nd ,,	4th day cult.; titre 10^{-3}	1 flask 10^{-5} 1 ,, 10^{-6} 1 ,, 10^{-2}	..	1 flask 10^{-1} 2 flasks neg.	3 flasks neg.	..	..
3rd ,,	3rd day cult.; titre 10^{-6}	3 flasks neg.	3 flasks neg.	1 flask 10^{-6} 1 ,, 10^{-5} 1 ,, 10^{-1}	..	..	..
4th ,,	5th day cult.; titre 10^{-5}	3 flasks neg.	..	3 flasks neg.	1 flask 10^{-1}	..	2 flasks 10^{-6}
5th ,,	5th day cult.; titre 10^{-6}	1 flask 10^{-1} 1 ,, 10^{-3} 1 ,, 10^{-2}	1 flask 10^{-1} 1 ,, 10^{-2} 1 ,, neg.	..	3 flasks neg.	..	..

TABLE II.—*Showing Second Series of Subcultures using Filtrate of Neuro-testicular Virus in Kidney Extract after One and Two Periods of Centrifugalization.*

(A) *Extract Spun Once for 30 Minutes at 10,000 r.p.m.*

Subcultures.	Virus used and titre.	Titre of cultures and days of incubation.				
		3rd day.	4th day.	5th day.	6th day.	7th day.
1st culture	N.T.V.; titre 10^{-6}	2 flasks 10^{-2}	2 flasks 10^{-2}	..	2 flasks 10^{-6}	2 flasks 10^{-6}
1st subcult.	6th day culture; titre 10^{-6}	3 flasks neg.	3 flasks neg.	..	1 flask 10^{-4} 2 flasks neg.	..
2nd ,,	8th day culture; titre 10^{-4}	1 flask 10^{-6} 2 flasks 10^{-1}	2 flasks 10^{-6} 1 flask 10^{-1}	..	3 flasks 10^{-1}	..
3rd ,,	4th day culture; titre 10^{-6}	..	2 flasks 10^{-6} 1 flask 10^{-5}	1 flask 10^{-6} 1 ,, 10^{-3} 1 ,, 10^{-1}	..	3 flasks 10^{-2}
4th ,,	4th day culture; titre 10^{-6}	3 flasks 10^{-1}	3 flasks 10^{-1}	1 flask 10^{-6} 1 ,, 10^{-4} 1 ,, 10^{-3}	..	..
5th ,,	5th day culture; titre 10^{-6}	..	1 flask 10^{-3} 2 flasks neg.	1 flask 10^{-6} 2 flasks 10^{-1}	3 flasks 10^{-1}	..
6th ,,	5th day culture; titre 10^{-6}	..	3 flasks neg.	3 flasks 10^{-2}	2 flasks neg. 1 flask 10^{-1}	..
7th ,,	5th day culture; titre 10^{-2}	3 flasks neg.	1 flask 10^{-5} 1 ,, 10^{-2} 1 ,, neg.	..	3 flasks neg.	..

(B) *Extract Spun Twice for 30 Minutes at 10,000 r.p.m.*

1st subcult.	4th day culture; titre 10^{-6}	..	3 flasks 10^{-6}	3 flasks 10^{-2}	1 flask 10^{-6}	1 flask 10^{-4} 1 ,, 10^{-1}
2nd ,,	4th day culture; titre 10^{-6}	3 flasks neg.	1 flask 10^{-2} 2 flasks neg.	1 flask 10^{-6} 1 ,, 10^{-3} 1 ,, 10^{-1}	..	..
3rd ,,	..	..	3 flasks neg.	1 flask 10^{-2} 2 flasks 10^{-1}	3 flasks neg.	..
4th ,,	5th day culture; titre 10^{-2}	..	3 flasks neg.	3 flasks neg.	3 flasks neg.	..

TABLE III.—*Showing Series of Seven Subcultures in Cell-free Medium using a Suspension of Elementary Bodies from Dermal Lapine as Seed.*

Subcultures.	Virus used and titre.	Titre of cultures and days of incubation. 2nd day.	3rd day.	4th day.	5th day.	6th day.	7th day.
Primary culture	E.B.S. 10^{-9}	2 flasks 10^{-6}	..	..	1 flask 10^{-5} 1 ,, 10^{-3} 1 ,, 10^{-4}	3 flasks 10^{-9}	..
1st subcult.	2nd day culture; titre 10^{-6}	1 flask neg. 2 flasks 10^{-1}	1 flask 10^{-2} 1 ,, neg. 1 ,, 10^{-3}	..	2 flasks 10^{-8} 1 flask 10^{-2}	..	..
2nd ,,	3rd day culture; titre 10^{-3}	2 flasks 10^{-8} 1 flask 10^{-3}	3 flasks 10^{-9}	..	1 flask 10^{-6} 1 ,, 10^{-4} 1 ,, 10^{-3}	..	..
3rd ,,	3rd day culture; titre 10^{-9}	3 flasks neg.	1 flask 10^{-8} 1 ,, 10^{-4} 1 ,, neg.	2 flasks 10^{-7} 1 flask 10^{-9}	..	..	..
4th ,,	4th day culture; titre 10^{-7}	..	1 flask 10^{-5} 1 ,, 10^{-3} 1 ,, 10^{-1}	3 flasks neg.	3 flasks neg.	..	..
5th ,,	3rd day culture; titre 10^{-5}	..	1 flask 10^{-5} 1 ,, 10^{-3} 1 ,, 10^{-2}	..	1 flask 10^{-9} 1 ,, 10^{-1}	1 flask 10^{-3} 2 flasks 10^{-1}	..
6th ,,	5th day culture; titre 10^{-9}	..	..	2 flasks 10^{-8} 1 flask 10^{-7}	1 flask 10^{-5} 1 ,, 10^{-3} 1 ,, 10^{-4}	3 flasks 10^{-1}	..
7th ,,	4th day culture; titre 10^{-5}	..	..	3 flasks neg.	1 flask 10^{-8} 2 flasks 10^{-4}	1 flask 10^{-3} 2 flasks 10^{-1}	..

flasks and the difficulty in carrying a series of subcultures to a satisfactory conclusion, it seemed possible that an explanation of these might be found on the ground of the technique employed in estimating the presence of virus. It was hoped, also, that an explanation might be found for the rapid and striking fall in titre in a set of culture flasks titrated after 3 days' incubation, for example, where after a longer period of incubation high titres were obtained. If multiplication had not taken place there were two possibilities which might account for the undeniable presence of virus at the conclusion of a series of subcultures. One of these was to assume that merely good survival of the original virus inoculum had been obtained in cell-free medium, and that the titre remained at a high level owing to an error introduced by the use of the dropping pipette used for titrations. In other words, the dilution factor in subcultures was modified by a technical error in titrations. The other possibility had also to do with the use of the dropping pipette, and assumed that repeated washing of the pipette had failed to remove the lymph used for the controls, and that the residual lymph appeared in the next set of titrations of cultures.

In order to test the first possibility of technical error a large number of parallel titrations were made with virus dilutions prepared in different ways. One set was made using the Dreyer dropping pipette, another using one 1 c.c. graduated pipette, and a third using a separate graduated 1 c.c. pipette for each dilution. All three sets were then inoculated in 0·2 c.c. amounts intradermally on one rabbit in order that the end-points might be strictly comparable. Many titrations of this kind were made, using elementary body suspensions, commercial lymph and neuro-testicular virus. A careful survey of the results showed that there was, on the whole, little difference in end-point, whatever method was used. In some cases the dropping pipette gave end-points one or two dilutions higher than the separate pipettes, in others identical end-points, and in some cases slightly lower ones. The same was true when a single 1 c.c. pipette was used. There was no difference in the results whether a single 1 c.c. pipette was used or the Dreyer dropping pipette.

A second method of testing was devised. This was simply to set up a culture in cell-free medium in the ordinary way, to make a series of subcultures in the medium at once and to carry out titrations at various stages in the series. Elementary bodies were used in one series as inoculum and, in the other, neuro-testicular virus. Titrations were made in parallel with the dropping pipette and separate 1 c.c. pipettes for each dilution. A typical result is shown in Table IV.

After a number of such experiments it was obvious that the Dreyer pipette did not produce an exaggerated end-point of existing virus, nor did its use give an erroneous appearance of multiplication of virus. In actual cultivation experiments it will be remembered, the deleterious effect on the virus of prolonged incubation is a factor to be considered, since, in the absence of multiplication, the titre of the virus falls gradually at incubator temperature.

The second possible error, that vaccine lymph was carried over in the dropping pipette from day to day, was then investigated. It has been pointed out that a considerable amount of water was forced through the pipette by washing on the tap immediately after making the final dilution of lymph.

TABLE IV.—*Showing the Effect on the Virus Inoculum of Simple Dilution in Series in Cell-free Medium and Titration by Dreyer Pipette and Separate* 1 *c.c. Pipettes.*

Dilutions of N.T. virus.	Original culture.		2nd subculture.		4th subculture.		7th subculture.	
	Dreyer pipette.	Separate pipette.	Dreyer pipette.	Separate pipette.	Dreyer pipette.	Separate pipette.	Dreyer pipette.	Separate pipette.
10^{-1}	++++	++++	++++	++++	+	+	—	—
10^{-2}	+++	+++	++	++	—	—	—	—
10^{-3}	+++	+++	+	+	—	—	—	—
10^{-4}	++	++	—	—	—	—	—	—
10^{-5}	+	+	—	—	—	—	—	—
10^{-6}	—	—	—	—	—	—	—	—
10^{-7}	—	—	—	—	—	—	—	—

TABLE V.—*Showing Parallel Titrations by Dreyer Dropping Pipette and Separate* 1 *c.c. Pipette of Separate Flasks in Subculture.*

Subcultures.	Virus used and titre.	Titre of cultures and days of incubation.			
		3rd day cultures.		4th day cultures.		5th day cultures.	
		Dreyer pipettes.	Separate pipettes.	Dreyer pipettes.	Separate pipettes.	Dreyer pipettes.	Separate pipettes.
Primary	E.B.S.	2 flasks 10^{-2}	10^{-2}	1 flask 10^{-3}	10^{-3}	1 flask 10^{-3}	10^{-3}
		1 flask 10^{-4}	10^{-5}	1 ,, 10^{-5}	10^{-7}	1 ,, 10^{-3}	10^{-3}
				1 ,, 10^{-6}	10^{-6}	1 ,, neg.	neg.
1st subcult.	4th day culture; titre 10^{-6}	1 flask neg.	$\mp$ 10^{-1}	3 flasks neg.	neg.	3 flasks neg.	neg.
		1 ,, 10^{-1}	10^{-1}				
		1 ,, neg.	neg.				

$\mp$ Denotes lesion of less than 5 cm. in diameter without induration.

This was followed by washing with saline, and the process repeated before the pipette was again used for making dilutions of cultures. It has seemed reasonable to assume that all virus must be removed from the pipette by this treatment. Even if some were left it must be enormously diluted and quite beyond the range of infectivity. It seemed advisable, however, to test this point carefully. The method used was to make a 1 in 10 dilution of commercial lymph, draw it up into the entire length of the pipette and allow it to run out slowly. The pipette was then washed on the tap and with saline in the manner ordinarily used and left on the bench overnight. The following morning it was again washed on the tap and with saline, a number of tubes were taken and, using the pipette, saline was dropped in after the manner of preparing a series of culture dilutions. 0·2 c.c. from each tube was injected intradermally in rabbits. A lymph control was used on each rabbit to test whether the animal was responsive to vaccinia virus. Readings were made from the third to the seventh day thereafter.

A number of such tests were made, using, in addition, neuro-testicular virus and a suspension of elementary bodies. Although the rabbits were susceptible to vaccinia, as shown by the lymph controls, the saline injections were entirely negative. It seemed clear, therefore, that if any virus had remained in the pipette after washing, the amount was too small to cause any reaction on the rabbits. It should be remembered on this point, that if residual lymph in the pipette were responsible for the appearance of extra-cultural virus, it must have been carried over at least once in testing each subculture. This is not in agreement with the results obtained in deliberate attempts to expel residual lymph into saline dilutions. It is inconceivable, also, that a titre as high as 10^{-6} could be obtained after so great dilution in view of the fact that a highly potent sample of lymph rarely exceeds a titre of 10^{-5}. Furthermore, it is difficult to explain on this basis a titre of 10^{-8} in the seventh subculture when, in the presumed absence of multiplication and survival, and after dilution through subcultures, the original virus inoculum must have become entirely inactive.

It was then decided to carry out a series of subcultures as before, but to make parallel titrations of each flask, using both the Dreyer dropping pipette and separate 1 c.c. pipettes for each dilution. It was hoped that in this way a conclusive answer might be obtained to the question why some series were successfully carried on whereas others failed, and why other investigators failed to obtain results similar to my own. The failure, for the purposes of the present investigation, has been assumed to be due to different methods of technique employed.

The method of preparing the medium and making of subcultures was identical in every way with that employed in the series outlined in Table III. The dilutions were made in parallel and inoculated on rabbits, so that the dilutions of each flask by both methods were tested on the same rabbit. In this way any differences observed could not be attributable to the variable reaction of rabbits to the same dose of virus, but must be due to the method of preparing the dilutions. Three separate series were attempted. Of these one became contaminated in the second subculture ; one showed a gradual diminution in titre in all flasks until the third subculture, which was entirely negative

by both methods of titration ; and in the third the virus failed to survive the first subculture. Table V shows the behaviour of the virus in the third series, and is included for the purpose of showing that whatever amount of virus is present in any culture flask it is practically the same irrespective of method of titration. It also indicates that success or failure in a series of subcultures does not appear to depend upon the technique of titration.

DISCUSSION.

That vaccinia virus, in common with many others, may be cultivated in the presence of living cells has been abundantly proved and needs no further comment. The question whether living cells are absolutely essential has received scant attention, in spite of its obvious importance in elucidating the nature of the filterable viruses. The problem is not one of degree, but of possibility ; for there can be no doubt that a virus grows better in the presence of living cells than in their absence. The accepted opinion now is that in the absence of living susceptible cells a virus not only fails to multiply, but also to survive for any considerable time at incubator temperature.

Evidence of multiplication of a virus is based upon three main conditions : a significantly higher titre in the incubated culture than in an unincubated control under otherwise identical circumstances ; the presence of a significant quantity of virus after sufficient dilution of the original inoculum through a series of subcultures to render it non-infective in the absence of multiplication; and a failure of the virus to survive in an unsuitable medium at incubator temperature. Under these conditions the experiments outlined in the present communication would seem to indicate that multiplication has occurred in certain individual flasks in certain series, and that in others the virus has neither multiplied nor survived. At best, growth of virus was not well maintained.

There are two possible interpretations of the data here presented. One is that the vaccinia virus has been cultivated with difficulty in a medium which does not provide ideal conditions for growth. It has been stated that the medium used was apparently free from whole, undamaged cells, and in this connection it may be argued that the medium in some flasks contained a small number of whole, living cells sufficient to account for multiplication of the virus in those flasks, while in others such cells were entirely absent, and so no multiplication could take place. It cannot be said with certainty that this may not have occurred, since filtration, which is the only completely reliable method available for eliminating single cells from the medium, was not used. It is significant, however, that other investigators have been unsuccessful in obtaining growth of virus in a similarly prepared medium, although the chances of similar distribution of occasional cells must have been equal.

There is no reason to believe that the strain of virus employed is an important factor, since similar results were obtained with both neuro-testicular vaccinia and with elementary body suspensions prepared from dermal lapine originating from commercial lymph. If adaptation to an unsuitable medium occurs it would seem to be uncertain.

The second possibility—that of technical error—has been discussed in

some detail. In so far as possible all surmisable sources of error have been investigated, and no evidence has been found to show that either occasional successes or failures in cultivation were attributable to the methods employed in measuring the amounts of virus present in the cultures or to the introduction of virus from an extracultural source.

That the virus present in cultures was, in fact, vaccinia was shown by neutralization tests with appropriate serum. It would seem, therefore, that conditions which may permit growth of vaccinia are occasionally present in certain individual flasks in a set containing "kidney extract" medium, but that such conditions are at present unknown and uncontrollable. Beyond this it is still impossible to go, and the vexing question whether the virus is able to reproduce itself in the absence of living susceptible cells must remain an open one.

SUMMARY.

The behaviour of vaccinia virus in a medium apparently free from whole living cells has been further investigated. A number of series of subcultures are presented in detail.

The difficulties encountered in previous experiments were again experienced. These are shown by the poor survival of the virus in large numbers of individual flasks, and the inability to secure either growth or survival of the virus in a number of subcultures.

Multiplication of the virus has apparently taken place in two of the series of subcultures here presented.

Possible sources of error in technique and interpretation have been carefully considered and investigated. But no source of error has been so far identified which would suggest that apparently successful subcultures, as judged by the titrations, may not be interpreted as genuine evidence of growth in a cell-free medium.

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ABBREVIATIONS USED IN REFERENCES.

The abbreviations given in the second edition of the 'World List of Scientific Periodicals' are now used in place of those given in the first edition. The chief difference is that adjectives are no longer given initial capitals in English.